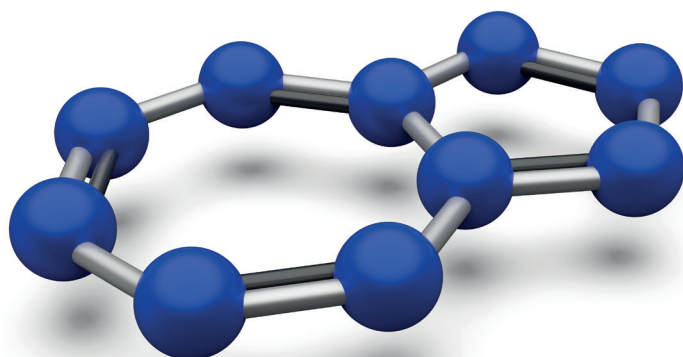


DISSERTATIONES SCHOLAE DOCTORALIS AD SANITATEM INVESTIGANDAM
UNIVERSITATIS HELSINKIENSIS

44/2018

TEPPO O. LEINO

Synthesis of Azulene-Based Compounds for Targeting Orexin Receptors



DRUG RESEARCH PROGRAM
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Synthesis of Azulene-Based Compounds for Targeting Orexin Receptors

Teppo O. Leino

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Pharmacy of the University of Helsinki, for public examination in Auditorium 2, Info Centre Korona, on August 16th 2018, at 12 noon.

Helsinki 2018

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Dissertationes Scholae Doctoralis Ad Sanitatem Investigandam Universitatis Helsinkiensis

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ISBN 978-951-51-4358-7 (paperback)
ISBN 978-951-51-4359-4 (PDF)
ISSN 2342-3161 (print)
ISSN 2342-317X (online)

Cover image by Lassi Leino

Hansaprint Oy
Helsinki 2018

“Genius is one percent inspiration and ninety-nine percent perspiration.”

Thomas A. Edison

Abstract

Orexin peptides, orexin-A and orexin-B, and orexin receptor 1 and orexin receptor 2 form the orexin signaling system. The most studied part of the orexin system is its key role in sleep-wake regulation, although it is linked also to other physiological functions, such as addiction and nociception. To date, a large number of orexin receptor antagonists have been developed with one, suvorexant, having reached the market in the treatment of insomnia. However, much less attention has been paid to the development of small-molecular agonists of the orexin receptors, and only a few are known in the literature. Agonists might be beneficial for patients with narcolepsy or certain types of cancers.

The aim of this thesis was to develop small molecules based on the azulene scaffold for targeting orexin receptors. Azulene is an unexplored ring structure in medicinal chemistry, however, it resembles other bicyclic aromatics such as indole and naphthalene, which are frequently found in drug molecules. The small number of existing general synthetic routes for azulenes possessing three or more substituents has most likely hindered the use of azulene-based compounds in medicinal chemistry. Due to this, the study was initiated by developing two different synthetic routes to access 1,3,6-trisubstituted azulenes.

In the developed methods, the azulene scaffold was first synthesized from simple, readily available and inexpensive starting materials. Then the scaffold was functionalized via versatile synthetic handles, such as a halogen atom or a formyl group, which allow a facile generation of compound series. The efficiency of the synthetic routes was demonstrated with test substances, which gave good overall yields.

The developed methods were used in the synthesis of azulene-based compounds, whose biological activity was assessed on the orexin receptors. The first series of compounds was based on the results from virtual screening of the library of 70 000 synthetically accessible azulene-based compounds. The second series was designed based on the results from the biological evaluation of the first series.

With this approach, novel azulene-based ligands for orexin receptors were identified. The two most promising binders had K_i values in the low micromolar range and five other compounds acted as weak orexin receptor agonists. In addition, compounds potentiating the response of orexin-A to OX_1 receptors in a concentration-dependent manner were discovered. These novel azulene-based compounds offer an interesting starting point for further development of antagonists, agonists and potentiators for orexin receptors.

Acknowledgements

This study was carried out at the Division of Pharmaceutical Chemistry and Technology, Faculty of Pharmacy, University of Helsinki in Finland during the years 2013–2018. A part of the study was performed at the Department of Chemistry, Durham University in United Kingdom in 2014, during my six months research visit. I acknowledge the Doctoral Program in Drug Research of the University of Helsinki for funding my doctoral studies.

First, I want to thank both my supervisors Doc. Erik Wallén and Prof. Jari Yli-Kauhaluoma for their inspiring scientific guidance and for giving me the opportunity for my doctoral studies. During the process they have always had time to discuss plans, synthesis and many other things related to the work. I also want to highlight that their enthusiasm for chemistry and inspiring teaching style had a significant impact on increasing my interest in organic chemistry, which led me to seek my way to JYK-group as a summer trainee during my undergraduate studies in 2010.

I am also grateful to Prof. Ian Baxendale for the possibility to visit his fantastic research group in Durham and the opportunity to meet all the great people there. I want to express my gratitude to the collaborators and co-authors, Prof. Jyrki Kukkonen, Dr. Henri Xhaard, Dr. Marcus Baumann, Ainoleena Turku, Lasse Karhu, Niklas Johansson, Lars Devisscher and Nina Sipari, who have been involved in azulenes and/or orexins during this study both in Helsinki and Durham. Special thanks to Ainoleena, who has had all the fun with me while writing the manuscripts. Furthermore, I want to acknowledge Prof. Jouko Vepsäläinen and Doc. Arto Liljeblad for reviewing this thesis.

For me it has been a sincere pleasure to work in the JYK-group for all these years and I want to thank all former and present colleagues, which I have had a privilege to work with. Especially I want to mention Mikko, Mikael, Laura, Leena, Raisa, Paula, Kristian, Riky, Gusse, Alexi, Rali, Irene, Ghada, Jayendra, Henri and Tommi for experiencing all the great moments in the lab, office, lunch breaks and conference trips. I have always been lucky enough to share the lab with colleagues, who agree with me that syntheses are working the best when listening to rock music. Additionally, I want to thank the almost-JYK-group-members Katja and Erkkä (Man of Harvard) for all their participation in everything.

I want to acknowledge my parents, Olli and Marja, who have always encouraged me on my studies. I also want to thank Lassi for his support and for designing the cover image of this thesis. Special thanks belongs also to all my friends, who have helped me to put the work aside every now and then. Finally, I am deeply grateful to my beloved wife and colleague Tiina, whose support has been irreplaceable during this process.

Helsinki, July 2018

Teppo Leino

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List of original publications

This thesis is based on the following publications:

- I Leino, T.O.; Baumann, M.; Yli-Kauhaluoma, J.; Baxendale, I.R.; Wallén, E.A.A. Synthesis of 1,3,6-Trisubstituted Azulenes. *J. Org. Chem.* **2015**, 80, 11513–11520. DOI: 10.1021/acs.joc.5b02271.
- II Leino, T.O.; Johansson, N.G.; Devisscher, L.; Sipari, N.; Yli-Kauhaluoma, J.; Wallén, E.A.A. Synthesis of 1,3,6-Trisubstituted Azulenes Based on the 1-Acyloxiazulene Scaffold. *Eur. J. Org. Chem.* **2016**, 5539–5544. DOI: 10.1002/ejoc.201600962.
- III Leino, T.O.*; Turku, A.*; Yli-Kauhaluoma, J.; Kukkonen, J.P.; Xhaard, H.; Wallén, E.A.A. Azulene-Based Compounds for Targeting Orexin Receptors. *Eur. J. Med. Chem.* **2018**, in press. DOI: 10.1016/j.ejmech.2018.07.040.
- IV Turku, A.*; Leino, T.O.*; Yli-Kauhaluoma, J.; Kukkonen, J.P.; Wallén, E.A.A.; Xhaard, H. Azulene-Based Compounds as Orexin Receptor Activating Agents. *Manuscript to be submitted*.

The publications are referred to in the text by their Roman numerals.

Publications related to, but not discussed in this thesis:

Turku, A.; Borrel, A.; Leino, T.O.; Karhu, L.; Kukkonen, J.P.; Xhaard, H. Pharmacophore Model To Discover OX₁ and OX₂ Orexin Receptor Ligands. *J. Med. Chem.* **2016**, 59, 8263–8275. DOI: 10.1021/acs.jmedchem.6b00333.

Karhu, L.; Weisell, J.; Turunen, P.M.; Leino, T.O.; Pätsi, H.; Xhaard, H.; Kukkonen, J.P.; Wallén, E.A.A. Stapled Truncated Orexin Peptides as Orexin Receptor Agonists. *Peptides* **2018**, 102, 54–60. DOI: 10.1016/j.peptides.2018.02.004.

* Equal contribution

Author's contributions

- I TOL planned the study, synthesized and characterized all compounds, and prepared the manuscript.
- II TOL planned the study, synthesized and characterized majority of the compounds. A few compounds were synthesized by NGJ and LD under supervision of TOL. TOL prepared the manuscript.
- III TOL contributed in planning of the study, and synthesized and characterized all compounds. AT carried out the computational and pharmacological parts of the study. TOL and AT prepared the manuscript with equal contribution.
- IV TOL contributed in planning of the study, and synthesized and characterized all compounds. AT carried out the computational and pharmacological parts of the study. TOL and AT prepared the manuscript with equal contribution.

Publications III and IV are also included in the dissertation of Ainoleena Turku.

Abbreviations

Ac	acetyl
BBB	blood-brain barrier
BINAP	2,2'-bis(diphenylphosphino)-1,1'-binaphthyl
Bn	benzyl
Bu	butyl
CHO	Chinese hamster ovary
CNS	central nervous system
COX-2	cyclooxygenase-2
Cy	cyclohexyl
DCM	dichloromethane
DMAP	4-(dimethylamino)pyridine
DMF	<i>N,N</i> -dimethylformamide
DMSO	dimethyl sulfoxide
DORA	dual orexin receptor antagonist
dpdb	dicyclohexylphosphano-2',6'-dimethoxybiphenyl
dppf	1,1'-bis(diphenylphosphino)ferrocene
EC ₅₀	half maximal effective concentration
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDT	ethane-1,2-dithiol
Et	ethyl
FDA	U.S. Food and Drug Administration
FLT-3	FMS-like tyrosine kinase 3
GABA	γ -aminobutyric acid
HMPA	hexamethylphosphoramide
HOBt	1-hydroxybenzotriazole
HOMO	highest occupied molecular orbital
HRMS	high resolution mass spectrometry
IC ₅₀	half-maximum inhibitory concentration
K _i	inhibition constant
LDA	lithium diisopropylamide
LUMO	lowest unoccupied molecular orbital
LUMO+1	second lowest unoccupied molecular orbital
Me	methyl
mRNA	messenger ribonucleic acid
mw	microwave
NIS	<i>N</i> -iodosuccinimide
NMR	nuclear magnetic resonance
OX ₁ R	orexin receptor 1
OX ₂ R	orexin receptor 2
Ph	phenyl
PKC	protein kinase C
PLC	phospholipase C

Pr	propyl
rt	room temperature
SAR	structure-activity relationship
SGLT2	sodium dependent glucose cotransporter 2
1-SORA	OX ₁ selective orexin receptor antagonist
2-SORA	OX ₂ selective orexin receptor antagonist
SSRI	selective serotonin reuptake inhibitor
Tf	trifluoromethanesulfonyl
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TMS	trimethylsilyl
TXA ₂	thromboxane A ₂

1 Introduction

1.1 The orexin system

1.1.1 Orexin peptides and receptors

Orexin peptides, orexin-A and orexin-B, form the orexin signaling system together with orexin receptor 1 (OX₁R) and orexin receptor 2 (OX₂R). Two research groups independently found the orexin peptides and their receptors in 1998. Sakurai et al. observed that the peptides they discovered are connected to feeding behaviour in rats.¹ Thus, they decided to name the peptides orexins after the Greek word *orexis*, meaning appetite. Around the same time de Lecea et al. found a mRNA sequence encoding the precursor of the orexin peptides in hypothalamus.² They called the resulting peptides as hypocretins: *hypo* since they were found from the hypothalamus, and *cretin* since they were partially analogous with the gut hormone secretin. Both terms orexin and hypocretin occur in the literature when referring to these peptides, and the term orexin is used in this thesis.

The orexin system is best known for its important role in sleep-wake regulation, but it has also been linked with other physiological functions, such as feeding behavior,¹ nociception³ and reward.⁴ The central role of the orexin system in sleep-wake regulation has been observed from its connections to narcolepsy. Originally this was noticed in narcoleptic dogs, which had a disruptive mutation in their OX₂R gene.⁵ Later studies with knockout mice have supported the connection between narcolepsy and dysfunction of the orexin system, and these studies have suggested that OX₂R has a more important role in type 1 narcolepsy than OX₁R.⁶⁻⁸ In addition, the link between narcolepsy and orexin system has been verified in humans: A majority of narcolepsy patients with cataplexy have very low orexin-A levels in cerebrospinal fluid, when compared to healthy subjects.⁹ Narcoleptic humans also lack most of the orexin-producing neurons.¹⁰

The effect of the orexin peptides is transmitted through the G protein-coupled orexin receptors, OX₁R and OX₂R, which have seven transmembrane domains (TM1-7) connected by three intra- and three extracellular loops, and the C-terminus is intracellular and the N-terminus extracellular. In humans OX₁R and OX₂R consist of 425 and 444 amino acids, respectively, and their complete sequences are 64% identical.¹ Binding of the orexin peptides to the receptors activates many signaling pathways.¹¹ One central response is an increase in the intracellular Ca²⁺ levels, which is caused by phospholipase C (PLC) mediated release of intracellular Ca²⁺ stores and protein kinase C (PKC) mediated Ca²⁺ influx across the plasma membrane. The measurement of Ca²⁺ elevation is widely used in orexin receptor activation studies.

The two orexin receptors are mainly located in various regions of the central nervous system (CNS), where the receptor subtypes are differentially expressed.¹² In addition, orexin responses have also been reported in the periphery.¹³ Interestingly, it has been

reported that certain types of cancer, such as colon cancer, have begun to express orexin receptors, whose activation would induce apoptosis.^{14,15} Thus orexin receptors are a potential target also for cancer treatment.

Orexinergic neurons are located in the lateral hypothalamic and nearby regions. Orexin peptides are enzymatically cleaved from prepro-orexin, which is the orexin precursor consisting of 131 amino acids.^{1,16} Orexin-A is a 33 amino acid long peptide containing two disulphide bridges between the cysteins (Cys6–Cys12, Cys7–Cys14), and pyroglutamic acid at the N-terminus (Figure 1). Orexin-B is the shorter one of the orexin peptides, consisting of 28 amino acids not bearing any bridging within the structure. Both orexin peptides have C-terminal amide groups. The NMR structures of the orexin peptides in a buffered aqueous environment have shown that orexin-A forms three α -helical segments (residues 6–9, 16–23 and 25–32) and orexin-B two (residues 7–19 and 23–28), respectively.^{17,18} Orexin-A can take either a bent or an extended conformation, whereas orexin-B only exists in a bent conformation.

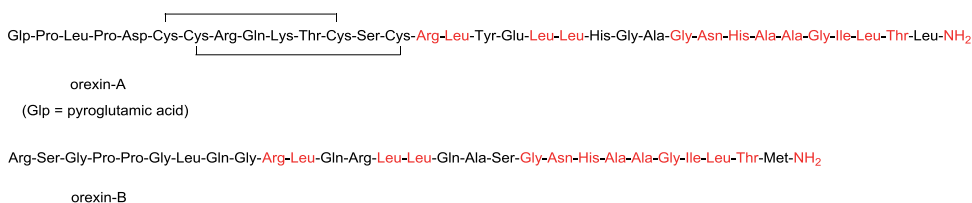


Figure 1 Primary structures of orexin-A and orexin-B. Conserved amino acids are highlighted with red color.

Orexin-A binds to both receptors with high affinity, whereas orexin-B has the same affinity to OX₂R as orexin-A, but the binding affinity to OX₁R is about ten times weaker. Furthermore, orexin peptides suffer from poor pharmacokinetic properties, typical to peptides. These include low blood-brain barrier (BBB) permeability,^{3,19} low bioavailability in oral administration,³ and rapid metabolism.²⁰

The essential binding interactions of orexin peptides to the orexin receptors have been investigated in mutation and truncation studies of the peptides. Truncation studies have suggested that the C-terminus of the orexin peptides is fundamental for the receptor activation, since both peptides can be shortened down to 19 amino acid residues from the N-terminus with only a small reduction in potency.^{21–23} Thirteen of these 19 amino acid residues are in fact identical in both peptides (highlighted with red color in Figure 1). Additionally, truncation from the C-terminus, or changing the primary amide to a carboxyl group, has resulted in the loss of activity of orexin-B.²²

The C-terminus of orexin-A consists mainly of amino acid residues with hydrophobic side chains, which cause one side of the α -helical C-terminus to be highly lipophilic (Figure 2).^{18,24} Three residues with hydrophobic side chain (Leu20, Ile30, Leu31) have been suggested to be significant for the receptor activation in the alanine point mutations with truncated orexin-A peptides.^{21,23,25} The activity has also been lost after replacing Thr32 with alanine. The corresponding residues exist in orexin-B as well, and point

mutations of these residues cause similar effect as in orexin-A.²² However, the results of the mutagenesis studies should be interpreted with caution, as the change in activity can be caused by a conformational change of the peptide, and not necessarily as a lack of the essential interaction between the receptor and the peptide.

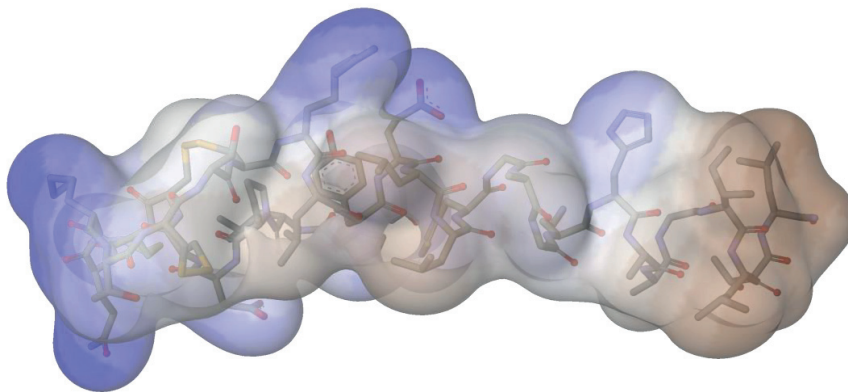


Figure 2 *Illustration of the hydrophobicity of orexin-A. Brown color represents hydrophobic areas and blue color hydrophilic. The intensity of the color depicts the strength of the property. (Figure kindly provided by L. Karhu)*

In addition to mutations in orexin peptides, orexin receptors have been mutated to reveal the binding site and important interactions between the orexin receptors and their ligands, both antagonists and endogenous orexin peptides.^{26–29} The binding site of small-molecular antagonists was confirmed by recently published crystal structures of the human OX₁ receptor with antagonists suvorexant (**1**) and SB-674042 (**2**) (Figure 3), and OX₂ receptor with suvorexant.^{30,31} Suvorexant binds to the orthosteric location, which is highly conserved in both receptor subtypes. In the crystal structures, Ser103/Thr111 and Ala127/Thr135 are the only amino acids, which differ in OX₁R/OX₂R, respectively, at a distance no further than 4 Å from suvorexant. Also **2** occupies the same area as suvorexant in OX₁R.

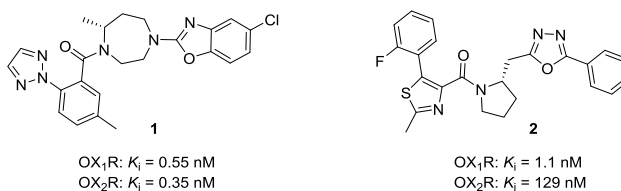


Figure 3 *Dual orexin receptor antagonist suvorexant (**1**) and OX₁R selective antagonist SB-674042 (**2**).*

To this date the orexin receptors have not been crystallized with orexin peptides, and even though mutagenesis and computational studies have given some hints, the binding site and the bioactive conformation of the peptides remain unclear.^{24,26,27} Identification of the binding sites and interactions is important in order to develop novel small-molecular ligands for the orexin receptors.

1.1.2 Ligands of the orexin receptors

The orexin receptors are attractive drug targets for developing both agonists and antagonists. After discovery of the receptors and their link to sleep-wake regulation, development of small-molecular ligands for the receptors has been of interest for the pharmaceutical industry. Several pharmaceutical companies have developed orexin receptor antagonists as a novel treatment for insomnia, which is a common sleep disorder and the current pharmacological treatment is mainly based on positive allosteric modulators of GABA_A receptor and sedative antidepressants.³² GABA_A modulators are intended only for short-term use and they have several side effects, such as amnesia, rebound insomnia and addiction. Also sedative antidepressants have many side effects depending on the main mechanism of action of the drug.

Orexin receptor antagonists can be divided in three groups based on their binding affinities: OX₁ selective orexin receptor antagonist (1-SORA), OX₂ selective orexin receptor antagonist (2-SORA) and dual orexin receptor antagonist (DORA). Development of 2-SORAs and DORAs as a new class of hypnotics has gained major attention until now, since the role of OX₂R in sleep-wake regulation is more significant than of OX₁R. Initially the focus of the development was in DORAs, and many DORAs, such as almorexant (**3**)^{33,34} and SB-649868 (**4**)³⁵, have shown to be effective in the treatment of insomnia in both animal models and humans (Figure 4). DORAs seem to promote physiological sleep resembling sleep architecture, unlike GABA_A receptor modulators, which tend to alter it.^{36,37} Possible induction of narcoleptic symptoms has been a major concern regarding the orexin receptor antagonists, but fortunately it seems the symptoms are not promoted.³⁴

The first orexin receptor antagonist suvorexant (**1**) was approved by the U.S. Food and Drug Administration (FDA) for sale for the treatment of insomnia in USA in 2014.³⁸ It is still the only one on the market, even though several other antagonists have been or are currently in clinical trials. In recent years there has been an increasing interest in developing 2-SORAs, since antagonism of OX₂ receptor alone has been reported to be sufficient in promoting sleep both in animals and humans.³⁹ However, the literature is still inconsistent regarding which compounds, 2-SORAs or DORAs, are optimal in promoting sleep with physiological sleep architecture.^{40,41} Inconsistencies between the studies could derive from differences in the test setups, different pharmacokinetic properties of the compounds, and possibly also other physiological targets of some compounds. The structures of three 2-SORAs, compounds JNJ-10397049 (**9**),⁴² MK-1064 (**10**)³⁹ and TCS-OX2-29 (**11**),⁴³ are presented in Figure 4.

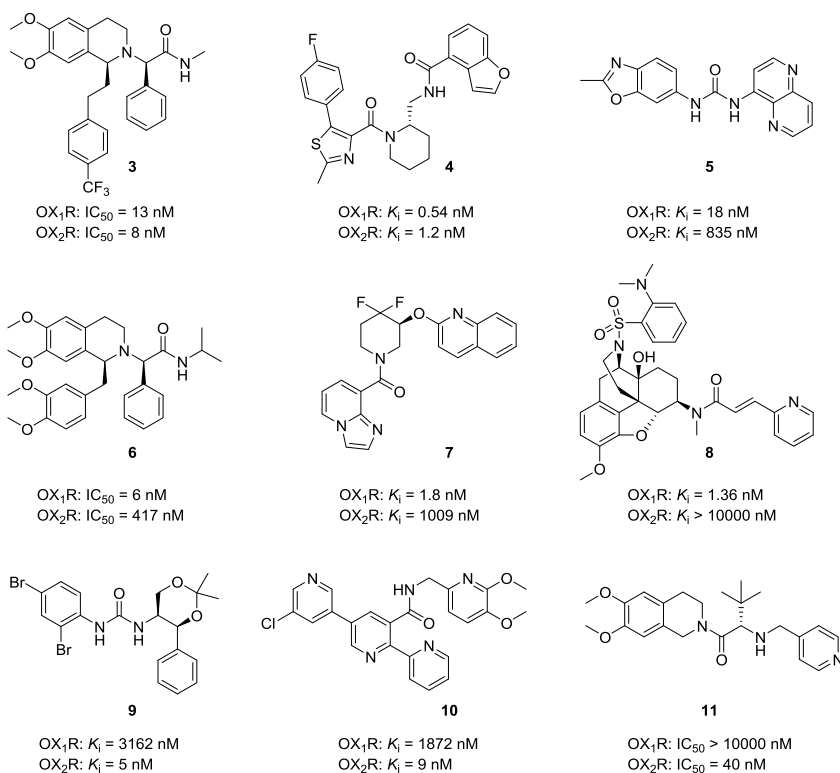


Figure 4 Structures and K_i values of dual orexin receptor antagonists **3** and **4**, selective OX_1 receptor antagonists **5–8** and selective OX_2 receptor antagonists **9–11**.

As described previously, orexin receptors are also involved in other physiological functions than sleep-wake regulation. Thereby, antagonists of the receptors might have other applications as well. 1-SORAs have been of particular interest in search for other possible targets, since they are largely ineffective to induce sleep.^{40,41} They might have potential in the treatment of various mental disorders, such as anxiety and panic, as well as addictive disorder. For example, the first known 1-SORA, SB-334867 (**5**) has blocked panic responses in rat panic model⁴⁴ and it has also reduced opioid withdrawal (Figure 4).⁴⁵ Another 1-SORA, ACT-335827 (**6**), has shown anxiolytic effects in rats without affecting motor coordination or cognitive functions.⁴⁶ Even though **5** is a widely used tool compound in research, it has disadvantageously several off-targets.⁴⁷ Recently, a highly potent and selective OX_1 receptor antagonist **7** has been developed to act as a more applicable tool compound.⁴⁸ Additionally, a morphinan-based series of potent and selective 1-SORAs has been published in 2017.⁴⁹ From this series, the most potent compound **8** has been shown to suppress morphine withdrawal symptoms in mice.

A common feature among the orexin receptor antagonist structures is two aromatic moieties, which are attached to each other with a linker usually containing an amide or a

urea functionality.^{50–52} Quite often the aromatic moieties are biaryls or fused bicyclic aryls, as shown in Figure 3 and Figure 4.

So far, when compared to antagonists, orexin receptor agonists have gained a minor attention in drug development. The best known agonists are the native peptide ligands, orexin-A and orexin-B, but the poor pharmacokinetic properties hamper their use as drugs or research tools. In addition to the orexin peptides themselves, truncated and/or mutated orexin peptides have been shown to be orexin receptor activating ligands. However, being peptides, they suffer from the same limitations as the orexin peptides, which emphasizes the need for novel and potent small-molecular orexin receptor agonists with good pharmacokinetic properties. Especially blood-brain barrier permeability is important for CNS agents. Small-molecular agonists could be useful as chemical probes in studying the function of orexin, particularly *in vivo*. Agonists might also show potential as therapeutic compounds in the treatment of daytime sleepiness, especially in narcolepsy, or certain types of cancer.

Narcolepsy is a chronic sleep disorder with excessive daytime sleepiness and unexpected sleep attacks.⁵³ In type 1 narcolepsy, patients also have cataplexy, which means sudden loss of muscle tone often triggered by strong emotions. Type 2 narcolepsy is then narcolepsy without cataplexy. Disorder in the orexin system has been connected to type 1 narcolepsy.⁹ Curative therapy for narcolepsy is still lacking and the current pharmacological treatment is only relieving symptoms by CNS stimulating drugs, such as modafinil and methylphenidate.⁵³ Additionally, certain antidepressants, like selective serotonin reuptake inhibitors (SSRIs), can be used in the treatment of cataplexy symptoms.

To date, only a few series of non-peptide orexin receptor agonists have been published. Nagahara et al. were the first ones to report a potent small-molecular OX₂ receptor agonist **12** in 2015 (Figure 5).⁵⁴ In search for an agonist they carried out a high throughput screening on OX₂R with ca 250 000 druglike compounds, followed by the synthesis of more than 1000 compounds. The sulphonamide group appeared to be essential for the receptor activation of **12**, since replacing the group with amide resulted in loss of activity.

Also the synthesis of **13**, a structural analogue of **12**, has been reported in the same publication (Figure 5).⁵⁴ Compound **13** appeared nearly as potent as **12** but has improved aqueous solubility and was therefore selected for further evaluation in animal models.⁵⁵ Narcolepsy symptoms of prepro-orexin knockout mice were relieved after peripheral administration of **13**. To further support the mechanism of action, no effect was observed in orexin receptor deficient mice, but in wild-type mice **13** promoted wakefulness. Together these results suggest that both narcolepsy and daytime sleepiness could be treated with orexin receptor agonists. However, a major drawback for the further development of **13** is its limited efficacy *in vivo*.

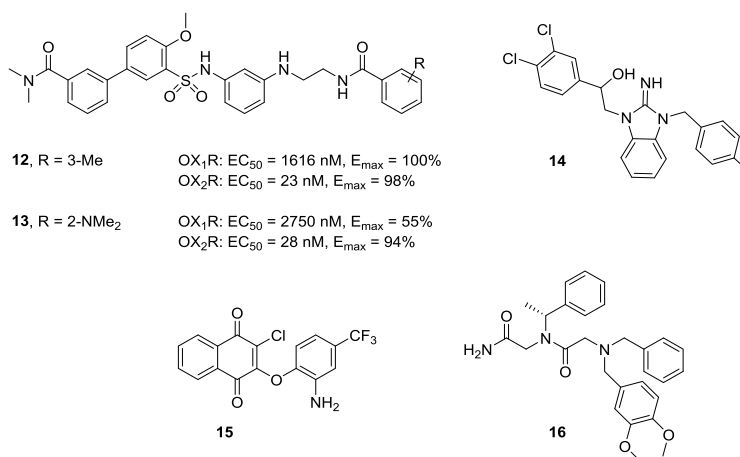


Figure 5 The structures of the reported orexin receptor agonists **12**–**15**. Compound **16** is an allosteric positive potentiator of the orexin receptor.

In addition, two series of orexin receptor agonists have been patented.^{56,57} The first series is based on the 1,3-dihydrobenzimidazol-2-imine scaffold, and the compounds of the second series are 2-(2-aminophenoxy)-3-chloronaphthalene-1,4-dione derivatives. Compounds **14** and **15** were the most promising ones from each series (Figure 5), but the agonist activity of these compounds has only been reported superficially. Later, Turku et al. have studied biological activity of the compound **14** in more detail.⁵⁸ They found compound **14** to be a weak orexin receptor partial agonist with cytotoxic properties, which restricts its use as orexin receptor agonist. Furthermore, Turku et al. have developed a pharmacophore model to discover orexin receptor ligands, based on which they have identified four weak partial agonists of the orexin receptors.⁵⁹

Positive orexin receptor potentiators are compounds enhancing the effects of endogenous orexin peptides. Potentiators could possibly be applied as a treatment of conditions caused by low levels of endogenous peptides. The potentiators would amplify the effect of the orexin peptides only when they are released physiologically, and therefore less side effects compared to agonists could be expected. Lee et al. have published the first series of allosteric orexin receptor potentiators identified in a microarray-based cell-binding screening and further optimization of peptoids, in which the aim was to find orexin receptor activators.⁶⁰ The best compound **16** showed approximately two-fold potentiation of EC₂₀ of orexin-A on orexin receptors.

1.2 Azulene

1.2.1 Structure and properties of azulene

Azulene (**17**) is an aromatic nonalternant hydrocarbon consisting of a five-membered ring fused to a seven-membered ring (Figure 6). Azulene is a structural isomer of naphthalene (**19**) as they both have the same molecular formula $C_{10}H_8$. However, the physicochemical properties of these two compounds differ considerably from each other. One indication of this is the color of the compounds: naphthalene is white, whereas azulene has a distinctive deep blue color. In fact, the name azulene is a derivative from Spanish word *azul*, which means blue.

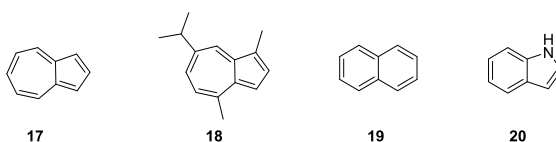


Figure 6 Azulene (**17**), guaiazulene (**18**), naphthalene (**19**) and indole (**20**).

The blue color of azulene derives from the exceptionally small energy difference between the ground state (S_0) and the first excited state (S_1) of the molecule, and unusually large energy gap between the first and the second excited states.^{61–63} The electron distribution of azulene differs remarkably between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) leading to the reduction in the repulsion forces between the electrons in these orbitals, which in turn explains the unusually small S_0 – S_1 energy gap (Figure 7). Furthermore, the probability of the electron location is similar in the HOMO and LUMO+1, which explains the increase in the energy of the second excited state. Substituents of the azulene scaffold influence the color, which can vary all the way from purple to red within the visible light spectrum, depending on the position and nature of the substituent. The color changes as a result from either stabilizing or destabilizing effect of the different substituents on HOMO, LUMO and LUMO+1, which results in changes in the energy gaps between the states. In addition to color, another unusual character of azulene is that it exhibits fluorescence from the second excited state ($S_2 \rightarrow S_0$).^{64,65}

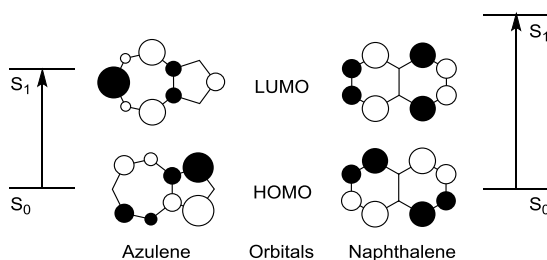


Figure 7 *Relative distribution of electron density for azulene and naphthalene in their ground state (S_0) and first excited state (S_1).*

The illustration of the HOMO of azulene also shows that the most probable location of the electrons is on the five-membered ring of azulene, while the seven-membered ring is electron-deficient. Thus, it can be considered that azulene is formed of a cyclopentadienide ion and a tropylium ion, which both are aromatic structures also on their own (Figure 8). This highlights the charge distribution between the rings, and it explains also why azulene has a dipole moment (1.08 D, measured in benzene).⁶⁶ Azulene can be considered as an isostere of indole (**20**, Figure 6), since both structures have an electron-rich five-membered ring fused to a larger ring, and a dipole moment between the rings.

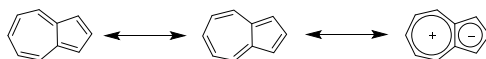


Figure 8 *Azulene has two Kekulé resonance structures as well as several others with separated charges. When the negative charge is drawn to be located on the five-membered ring and the positive charge on the seven-membered, azulene is formed of two fused aromatic structures: a cyclopentadienide ion and a tropylium ion.*

The electron distribution of azulene affects its reactivity. The electron rich five-membered ring readily reacts with electrophiles at the 1- and 3-position,⁶⁷ and these positions can be functionalized using electrophilic aromatic substitution reactions, such as Vilsmeier–Haack formylation and Friedel–Crafts acylation.^{68,69} However, the regioselectivity of these reactions between the 1- and 3-position is usually poor. Compared to the 1- and 3-position functionalization of the 2-position is more challenging, although it can be substituted with boronic acid pinacol ester in an iridium-catalyzed reaction.⁷⁰ The boronate group can be then converted to many other functionalities.^{70–72}

The reactivity of the five-membered ring is also important from the metabolic point of view, since, as expected, it is prone to enzymatic oxidation. In the metabolic studies of azulene in rats, the sulphate conjugate of 1-hydroxyazulene has been identified as the main metabolite in urine.⁷³ Interestingly, 1-hydroxyazulene is extremely unstable compound,⁷⁴ whereas 2-hydroxyazulene is stable and shows keto-enol tautomerism.⁷⁵ In order to prevent the undesired metabolic activity of azulene in medicinal chemistry applications, it

might be beneficial to substitute either both 1- and 3-positions, or at least one of these with a deactivating group.

The electron-deficient seven-membered ring of azulene reacts with nucleophiles at the 4-, 6- and 8-position,^{76–78} and also other reaction types to substitute these positions have been reported.^{79,80} Often these reactions either suffer from the lack of regioselectivity or they require certain substitution on the azulene scaffold. The 5- and 7-position of azulene are also nucleophilic to some extent, since they have been reported to react with electrophiles after the more nucleophilic 1- and 3-positions have already been substituted.^{81,82} Thus, in many cases the most convenient way for substitution of the seven-membered ring is to introduce the desired substituent in certain position already in the synthesis of the azulene ring, as described more in detail in the next section.

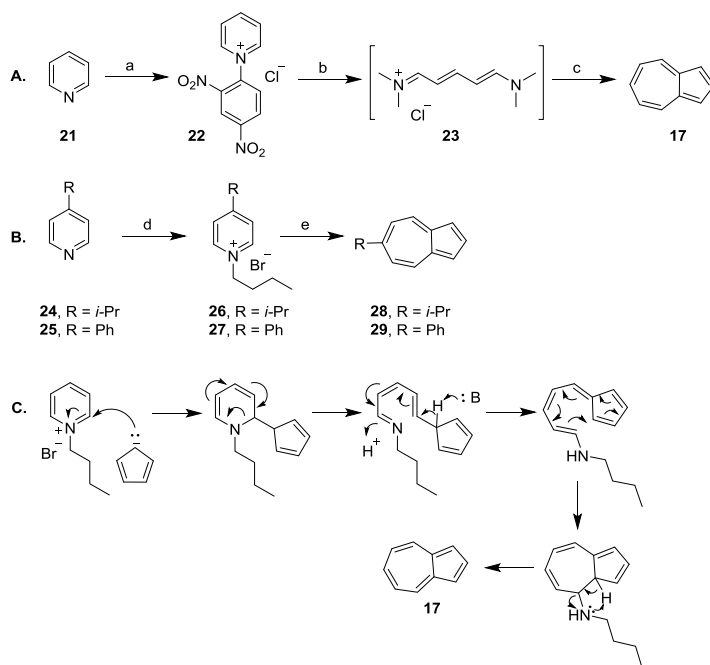
1.2.2 Synthesis of the azulene ring

Although azulene is a commercially available compound, its use in synthetic chemistry is limited by the relatively high price as well as the fact that the seven-membered ring of azulene is difficult to selectively substitute without a suitable synthetic handle. The natural product guaiazulene (**18**, Figure 6) is commonly used as starting material, since it is readily available and inexpensive. Even though the methyl and isopropyl groups of guaiazulene can be exploited by substituting the seven-membered ring,⁸³ on the other hand they limit the substitution pattern of azulene derivatives. Since chemical suppliers can provide only few other, relatively expensive substituted azulenes, in the synthesis of azulene derivatives the azulene ring is usually constructed from simpler starting materials. The formation of the bicyclic ring structure also allows introduction of various substituents as synthetic handles for further functionalization of the azulene scaffold. Certain important synthetic methods for azulene ring formation are described herein. Also the reaction mechanisms of the methods used in our study are provided.

In 1955 Ziegler and Hafner reported the first synthetic method for forming the azulene ring without any dehydrogenation step.^{84–87} The synthesis begins from the formation of (2,4-dinitrophenyl)pyridinium chloride (**22**) from pyridine (**21**) and 1-chloro-2,4-dinitrobenzene (Scheme 1A). In the next step the pyridinium ring is opened with an amine derivative and when using dimethylamine, the intermediate **23** is obtained. The intermediate **23** reacts with cyclopentadienyl anion, which after thermal cyclization gives azulene (**17**). The method allows introduction of various hydrocarbon substituents in the seven-membered ring when using variable pyridine derivatives as starting materials.

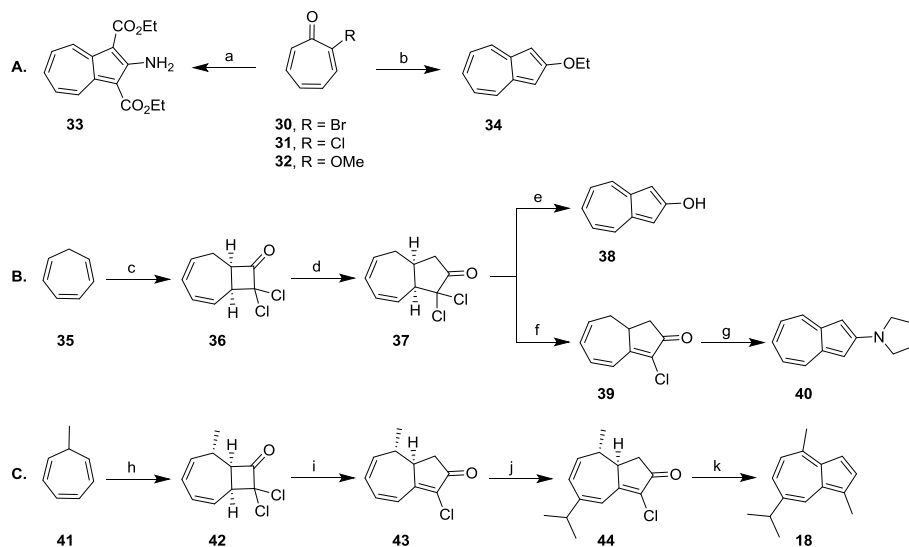
Modified reaction conditions for the Ziegler–Hafner method have been reported in the literature. An interesting modification utilizes alkyl pyridinium derivatives (Scheme 1B).^{88–90} Alkyl pyridinium readily reacts with cyclopentadienyl anion and no additional amine is needed for the ring opening. After thermal cyclization the azulene derivatives are obtained. The proposed reaction mechanism for the azulene ring formation is presented in Scheme 1C. The yields of the Ziegler–Hafner azulene synthesis vary a lot depending on the used pyridine derivatives and the reaction conditions. Bauer and Müller-Westerhoff

have modified the Ziegler–Hafner method to synthesize 6-dimethylaminoazulene from 4-pyrone instead of using pyridine as a starting material.⁹¹



Scheme 1 Two modifications of the Ziegler–Hafner azulene synthesis (A and B) and the proposed reaction mechanism (C). Reagents and conditions: (a) 1-Chloro-2,4-dinitrobenzene, 90 °C, 4 h; (b) Me₂NH, pyridine, 0 °C, 20 min, rt, 12 h; (c) i) Cyclopentadiene, NaOMe, ii) 125 °C, 4 d, 51–59% from **21**; (d) 1-Bromobutane, EtOH, reflux, overnight, 68% (**26**), 98% (**27**); (e) Cyclopentadiene, NaH, DMF, rt, 1 h, reflux, 3 h, 66% (**28**), 81% (**29**).

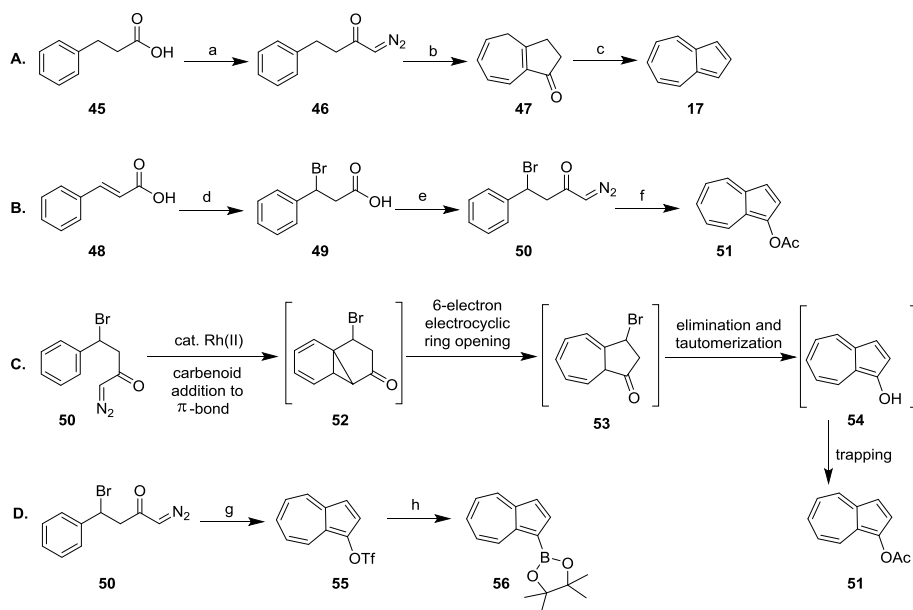
The azulene ring can also be synthesized from unsaturated seven-membered rings, such as tropone and cycloheptatriene derivatives. 2-Halo- and 2-methoxytropones react with malonic acid derivatives to form 1,2,3-trisubstituted azulenes, such as **33**, in the presence of a base (Scheme 2A).^{92,93} These substituents can also be removed, if needed. Additionally, substituents, such as a bromine atom or an alkyl group, can be introduced to the seven-membered ring with this method, starting from substituted 2-halotropones.⁹⁴ However, the yields are low and substituted 2-halotropones are rarely commercially available. Tropones have also been shown to react with prop-2-enylidetriphenylphosphorane derivatives to form the azulene ring, the synthesis of **34** from **31** as an example.⁹⁵



Scheme 2 Synthesis of the azulene scaffold from tropone (A) and cycloheptatriene (B and C) derivatives. Reagents and conditions: (a) Ethyl cyanoacetate, NaOEt, EtOH, rt, ca 70% from **31**; (b) (2-Ethoxyallylidene)triphenyl- λ^5 -phosphane, DMSO, rt, 12 h, 60% from **31**; (c) Trichloroacetyl chloride, Zn/Cu, Et₂O, rt, 4 h, 58%; (d) CH₂N₂, MeOH/Et₂O, rt, 30 min, 85%; (e) LiCl, DMF, 110–120 °C, 6 h, 73%; (f) Et₃N, THF, rt, 9 h, 95%; (g) Pyrrolidine, PhH, reflux, 12 h, 75%; (h) Trichloroacetyl chloride, Zn/Cu, POCl₃, Et₂O, rt, 20 h; (i) i) CH₂N₂, MeOH/Et₂O, 0 °C → rt, 35 min, ii) DMF, rt, 12 h, 45% from **41**; (j) i) $i\text{Pr}_2\text{Zn}$, CuOTf, HMPA, TMSCl, –100 °C, 6 h, ii) PhSeCl, –50 °C → 0 °C, 2 h, iii) H₂O₂, pyridine, 0 °C, 1 h, 62%; (k) i) NaBH₄, MeOH/CeCl₃, 0 °C, 1.5 h, ii) Burgess reagent, THF, 0 °C, 1 h, iii) p-Chloranil, rt, 24 h, iv) MeB(OH)₂, Pd(OAc)₂, dpdb, K₃PO₄, PhMe, 100 °C, 24 h, 53%.

Cycloheptatriene (**35**) reacts with dichloroketene, prepared *in situ* by treating trichloroacetyl chloride with activated zinc, to form intermediate **36** (Scheme 2B).⁹⁶ The four-membered ring of **36** is expanded to a five-membered ring with diazomethane. 2-Hydroxyazulene (**38**) is obtained after heating compound **37** in the presence of a lithium salt. The intermediate **37** can also be treated in other conditions to obtain various azulenes, such as 1-(azulen-2-yl)pyrrolidine (**40**) from the treatment of **37** with trimethylamine followed by pyrrolidine. Carret et al. have employed the method for 7-methylcycloheptatriene (**41**) as starting material (Scheme 2C).⁹⁷ They have also modified the final steps of the route, which has allowed substitution of the 1- and 7-position of azulene.

The seven-membered ring of azulene can also be prepared from benzene ring using Büchner's ring expansion method. Based on this, Scott et al. have developed a method for azulene synthesis, in which the bicyclic compound **47** is synthesized from the diazoketone **46** in the presence of a copper(I) catalyst (Scheme 3A).⁹⁸ However, azulene (**17**) is obtained in the following dehydrogenation step only in moderate yield.



Scheme 3 Two ring expansion-annulation strategies for the synthesis of azulenes (A and B). The reaction mechanism to form 1-acetoxyazulene (**51**) from the diazo ketone **50** (C). Conversion of a triflate group to a boronate group (D). Reagents and conditions: (a) i) SOCl_2 , reflux, 2 h, ii) CH_2N_2 , 0°C , 2 h, rt, 1 h, 97%; (b) CuCl , 80°C , 8 h, 52%; (c) P_2O_5 , $\text{CH}_3\text{SO}_3\text{H}$, 60°C , 6 h, 30–50%; (d) HBr-SiO_2 , DCM, rt; (e) i) $(\text{COCl})_2$, PhH , 65°C , ii) CH_2N_2 , Et_2O , $0^\circ\text{C} \rightarrow \text{rt}$, 69% from **48**; (f) i) $\text{Rh}_2(\text{t-BuCO}_2)_4$, DCM, rt, 45 min, ii) DMAP, Ac_2O , rt, 5 min, 64–72%; (g) i) $\text{Rh}_2(\text{t-BuCO}_2)_4$, DCM, rt, 45 min, ii) DMAP, Tf_2NPh , rt, 10 min (h) 4,4,5,5-tetramethyl-1,3,2-dioxaborolane, $\text{Pd}(\text{OAc})_2$, (*o*-biphenyl) PCy_2 , Et_3N , THF, rt, 16 h, 45–51% from **50**.

Kane et al. have modified the method reported by Scott et al. (Scheme 3B and C).⁹⁹ The synthetic route begins from cinnamic acid (**48**) with a hydrobromination reaction followed by the preparation of the diazo ketone **50**. The bromo ketone **53** is obtained after exposing of the diazo ketone to the rhodium(II) catalyst, and the subsequent elimination reaction in the presence of DMAP gives 1-hydroxyazulene (**54**). Since **54** is extremely unstable,⁷⁴ acetic anhydride has been used to trap the hydroxy group to give the stable ester **51**. Instead of acetic anhydride Kane et al. have shown that the hydroxy group can also be trapped with *N*-phenyl-bis(trifluoromethanesulfonylimide) to give triflate derivative **55** (Scheme 3D). However, the triflate **55** has also been characterized to be unstable, but it can be converted to more stable pinacol boronate ester **56**.¹⁰⁰ Both functionalities can be used in Suzuki cross-coupling reactions.

The advantage of this method is that cinnamic acid derivatives can be easily prepared from various benzaldehydes using Knoevenagel condensation, and when varying the substituents on the benzaldehydes, the final azulenes possess these substituents in the seven-membered ring. Kane et al. have shown that the method tolerates a wide range of

substituents, such as halogens, nitro and alkyl groups, in the phenyl ring. However, the introduction of both a halogen atom at the seven-membered ring and a triflate group at the 1-position lead to selectivity problems in Pd-mediated coupling reactions, since both functionalities are equally reactive under Suzuki coupling conditions.¹⁰⁰

In addition to the synthetic routes described above, other approaches are reported in the literature.^{101–104} Altogether, several methods for the synthesis of the azulene ring have been developed to date. Advantages and restrictions vary between the methods. Low overall yields, toxic and explosive reagents (e.g. diazomethane), availability and price of starting materials or difficulties to introduce substituents to seven-membered ring during the azulene scaffold formation are the major limitations in the previously described methods.

1.2.3. Azulene scaffold in medicinal chemistry applications

The azulene ring has not been intensively studied in medicinal chemistry so far, unlike its structural isomer naphthalene and its isostere indole.¹⁰⁵ The antiulcer agent egualen sodium (**57**) is the only azulene-based drug on the market,¹⁰⁶ and also some sulphonamide derivatives of guaiazulene, such as **58** and **59**, have shown antiulcer activity (Figure 9).¹⁰⁷ Egualen sodium has also been used as a starting point in the development of azulene-based thromboxane A₂ (TXA₂) receptor antagonists as it has shown some TXA₂ antagonist activity.^{108–110} As a result of the optimization process, **60** was found to be a potent TXA₂ receptor antagonist without partial agonist activities.

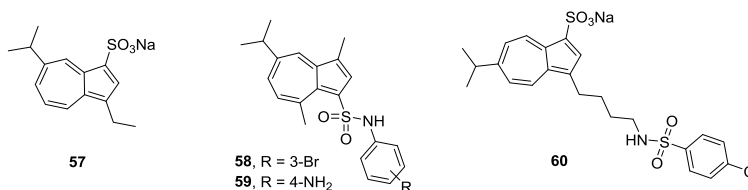


Figure 9 Azulene derivatives with antiulcer activity: Egualen sodium (**57**) and sulphonamide derivatives of guaiazulene **58** and **59**. Compound **60** is TXA₂ receptor antagonist.

The azulene ring has been used as a bioisostere in medicinal chemistry applications. In this approach, compounds with known biological activities have been modified by replacing the unsaturated rings of the compounds with azulene (Figure 10). Löber et al. have studied the use of azulene as an arene bioisostere in the development of novel dopamine D₄ receptor ligands for the treatment of erectile dysfunction.^{111,112} They have chosen dopamine receptor binding azaindole derivatives, such as **61**, as the starting point, and used azulene instead of the azaindole structure. Compound **62** showed subnanomolar binding and partial agonism to the D₄ receptor. In a further study they focused on functionalization of the 3-position of **62**, and in *in vivo* studies compound **63** induced penile erection in rats.¹¹²

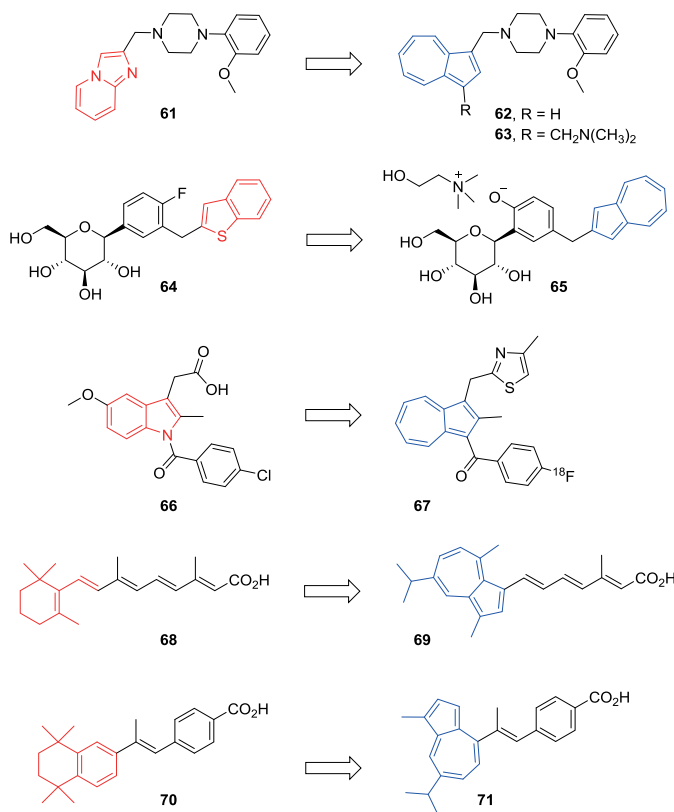


Figure 10 Utilizing azulene ring as a bioisostere in medicinal chemistry. The unsaturated ring, which is replaced by azulene, is marked in red color.

The antidiabetic agent ipraglifozin (**64**) is a C-glucoside-based sodium-dependent glucose cotransporter 2 (SGLT2) inhibitor developed by pharmaceutical companies Astellas and Kotobuki.¹¹³ The same companies have also investigated azulene derivatives of ipraglifozin by replacing the benzothiophene with azulene for the same target.¹¹⁴ The most potent azulene-based compounds have shown SGLT2 inhibition at low nanomolar concentrations. Furthermore, the best compound **65** induced strong and sustained antihyperglycemic effect in diabetic rodents after oral administration.

Azulene derivatives have also been developed for cancer imaging. Cyclooxygenase-2 (COX-2) overexpression has been reported in several human cancer cell lines, such as breast¹¹⁵ and stomach cancers.¹¹⁶ Inspired by this, Nolting et al. have studied a known indole-based COX-2 inhibitor, indomethacin (**66**), and synthesized indomethacin like ¹⁸F-labeled azulene derivative **67** as a radioactive COX-2 probe.¹¹⁷ In addition to cancer imaging, azulenes have been studied for their anticancer properties. Anticancer properties of retinoic acid (**68**) and its phenyl derivatives (e.g. **70**) have been of interest for Asato et al., who have replaced a part of these molecules with azulene derivatives to form new compounds, such as **69** and **71**. The resulting azulenic retinoids and retinobenzoic acid

analogs showed anticancer activity in the *in vitro* mouse fibroblast C3H/10T1/2 cell bioassay.¹¹⁸ However, the azulenic retinoids were less potent than **70**.

Other azulene-based compounds with anticancer properties are azulenes with oxindole and azaoxindole moieties, which inhibited FMS-like tyrosine kinase 3 (FLT-3) at low nanomolar concentrations making these compounds potential starting points in the development of a new class of antileukemia agents.¹¹⁹ Furthermore, azulene-indoles have shown *in vitro* activity against different human cancer cell lines.¹²⁰ The structures of the most promising compounds from these studies, **72** and **73**, are presented in Figure 11.

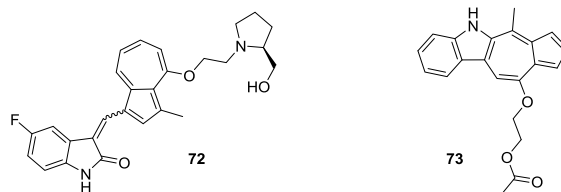


Figure 11 Azulene-based compounds with anticancer activities.

The natural product guaiazulene is used as an additive in cosmetics, such as lotions, although there is some evidence for its cytotoxic effects.¹²¹ Guaiazulene (**18**) and its derivative, stilbazulenyl nitron (74) have been reported to have antioxidant properties.^{122,123} The amidine derivative of guaiazulene **75** has inhibited arterial contractions *in vitro* in isolated pig coronary arteries.¹²⁴ The mechanism of action has been partially explained by the inhibition of L-type Ca^{2+} -channels and protein kinase C. Another naturally occurring azulene derivative, chamazulene carboxylic acid (**76**), has an anti-inflammatory activity,¹²⁵ likely due to its structural resemblance to the known non-steroidal anti-inflammatory drug naproxen (**77**). Finally, some azulene-based compounds have acted as antimicrobial agents in *in vitro* studies.^{126,127}

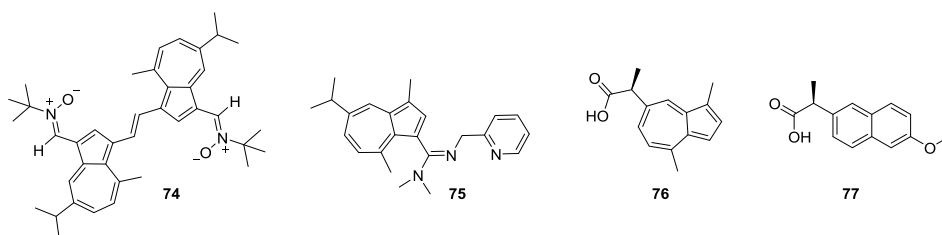


Figure 12 Azulene derivatives with various biological activities and the structure of naproxen (**77**).

To conclude, azulene can be considered as an interesting ring structure in drug discovery, even though it has not been widely applied so far. Inscrutability can also be considered as an advantage, since novel azulene-based compounds are most likely still patentable, which is vital in drug development. Small amount of available synthetic routes to access azulenes

possessing three or more substituents is one reason that limits the use of azulenes in medicinal chemistry. Due to this there is a need for the development of general synthetic protocols towards multiply substituted azulenes. Novel synthetic approaches could enable making series of azulene-based compounds for discovering new interesting bioactivities.

2 Aims of the study

The aim of the study was to develop small molecules for targeting orexin receptors, and in particular, to identify orexin receptor agonists. Mimicking of the C-terminus of orexin-A, which has been shown to be essential for the activation of the orexin receptors, was used as an approach in search for new orexin receptor ligands. The hydrophobic, bicyclic azulene skeleton was reasoned to resemble the lipophilic side of the α -helical C-terminus of orexin-A. Many known orexin receptor ligands, especially antagonists, were noticed to contain biaryls or fused bicyclic aryls, which suggests the orthosteric binding site of the receptor could form beneficial interactions with azulene as well. In addition, azulene is scarcely reported ring structure in medicinal chemistry, although it closely relates to other bicyclic aromatics frequently found in drug molecules. Thus, the study focused on investigating the potential of azulene as a scaffold for the orexin receptor targeting compounds.

The more specific aims of the study were:

- To develop general regioselective synthetic protocols to access trisubstituted azulenes (Publications I and II).
- To design and synthesize azulene-based compounds for targeting orexin receptors and study their activity in *in vitro* (Publication III).
- To further optimize azulene-based orexin receptor ligands to find compounds with enhanced agonist activity to orexin receptors (Publication IV).

3 Results and discussion

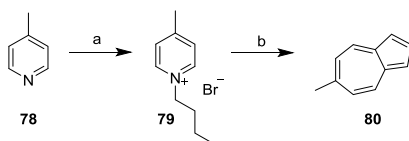
3.1 Synthesis of 1,3,6-trisubstituted azulenes

This study was initiated by developing general synthetic routes to access different 1,3,6-trisubstituted azulenes. We aimed at developing methods, which would allow the regioselective substitution of the azulene scaffold with the use of synthetic handles in the different substitution position. This approach enables an easy generation of compound series for biological activity testing. Various reasons supported focusing on the 1-, 3- and 6-positions of the azulene scaffold. First, the 1-, 3- and 6-positions are on different sides of the azulene scaffold. Second, the 1- and 3-positions are the most reactive ones of the azulene ring, and in order to enhance the stability of azulene derivatives, metabolic stability as an example, at least one of these positions should be substituted. Finally, the substitution of the seven-membered ring at the 6-position gives symmetrical azulene, in which the 1- and 3-position are identical and selectivity problems are not expected between these positions.

3.1.1 Synthesis of the azulene scaffold

As the selective substitution of the seven-membered ring of azulene is problematic without a suitable synthetic handle, it is advantageous to introduce a synthetic handle to the ring already during the formation of the azulene scaffold. Due to this, we first decided to investigate synthetic methods for construction of the azulene scaffold with suitable substituents on its 6-position. We chose 6-methylazulene (**80**) as the key intermediate for the first synthetic route (Publication I), since the methyl group can be further converted to other functionalities.¹²⁸ 6-Methylazulene has been previously synthesized from 1-butyl-4-methylpyridinium bromide (**79**) and sodium cyclopentadienide with the Ziegler–Hafner method.⁸⁸

First, amine alkylation of 4-methylpyridine (**78**) with 1-butybromide gave **79** in quantitative yield (Scheme 4). In the following step of the reaction 6-methylazulene was obtained in 23% yield, which was comparable with the 27% yield reported in the literature.⁸⁸ Several side products were formed in the reaction and they were difficult to separate from the product. Due to this we investigated whether the reaction conditions could be further optimized by varying concentration, solvent, base, temperature, reaction time, and heating regime using either conventional or microwave heating. The studied methods and yields are shown in Table 1. The best combination of base and solvent appeared to be NaH and DMF. The use of other bases and solvents resulted in lower yields, likely due to incomplete deprotonation of cyclopentadiene and reduced solubility of the pyridinium salt. However, rapid heating to high temperatures with microwaves and lowering the concentration improved the yields. The increased temperature also shortened the reaction time significantly.



Scheme 4 *Synthesis of 6-methylazulene. Reagents and conditions: (a) 1-Bromobutane, EtOH, reflux, overnight, quant.; (b) Base, cyclopentadiene, 2.5–64% (Table 1).*

Table 1 *Conditions used in the optimization of 6-methylazulene synthesis.*

	Temperature/time	Base	Solvents	c (M) ^a	Scale (mmol)	Yield (%)
1	rt, 1 h + reflux, 3 h	NaH	DMF	1.0	10	23
2	rt, 1 h + reflux, 3 h	NaOH	MeOH/THF 1:20	1.0	10	3.5
3	rt, 1 h + reflux, overnight	NaOMe	MeOH	1.0	10	2.5
4	rt, 1 h + reflux, 3 h	NaH	THF	1.0	10	6.0
5	rt, 1 h + reflux, 3 h	NaH	EtOH/THF 1:4	0.80	10	2.5
6	rt, 1 h + reflux, 3 h	NaH	DMF	0.50	10	44
7	rt, 5 min + mw, 170 °C, 30 min	NaH	DMF	0.25	2.5	57
8	rt, 5 min + mw, 152 °C, 30 min	NaH	DMF	0.25	2.5	45
9	rt, 5 min + mw, 200 °C, 30 min	NaH	DMF	0.25	2.5	59
10	rt, 5 min + mw, 170 °C, 30 min	NaH	DMF	0.10	1.0	64
11	rt, 5 min + mw, 170 °C, 30 min	NaH	DMF	0.050	0.50	52
12	rt, 10 min + mw, 170 °C, 30 min	<i>n</i> -BuLi	Hex/THF/DMF 1:1:2	0.25	2.5	25
13	rt, 15 min + mw, 170 °C, 30 min	<i>n</i> -BuLi	Hex/DMF 2.5:7.5	0.25	2.5	38
14	rt, 15 min + mw, 170 °C, 30 min	<i>n</i> -BuLi	Hex/DMF 1:9	0.10	1.0	60

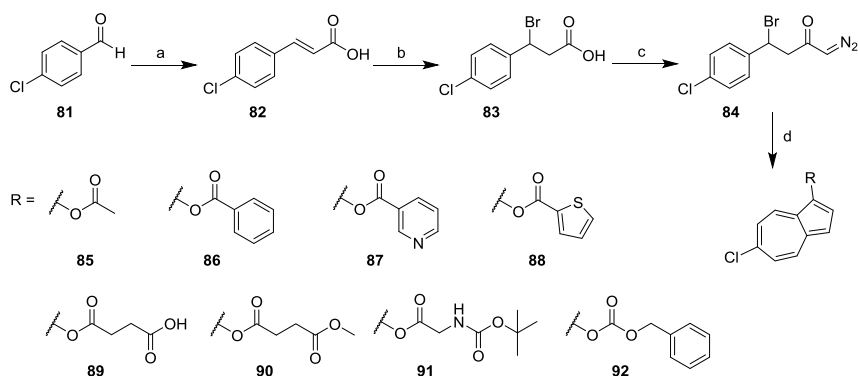
^a Concentration of 1-butyl-4-methylpyridinium bromide.

After optimizing the reaction conditions, the possibility to scale-up the synthesis of 6-methylazulene was evaluated. Entry 9 was chosen as the starting point for the scale-up process. As the volume of microwave vials is limited, the reaction mixture was divided into ten separate microwave vials (10–20 mL) and they were run sequentially using the robotic instrumentation. After microwave heating, the contents of the vials were combined into two batches for workup and further into one for flash chromatography. In this way, the 50 mmol scale was reached with an overall yield of 63% corresponding 4.5 g of pure 6-methylazulene. Additionally, we noticed that the heating time could be shortened from 30 min to 15 min without affecting the purity or the yield.

We also tried to synthesize 6-haloazulene with the same method as 6-methylazulene by changing the starting material from 4-methylpyridine to 4-halopyridine. Unfortunately, these attempts failed. As a halogen atom is a versatile synthetic handle and can be exploited in many palladium-mediated coupling reactions,^{100,129–133} we decided to study an alternative method to furnish an azulene ring with halogen substituent. A few synthetic routes for 6-haloazulene derivatives can be found in the literature, but most of them include multiple steps with low overall yields.^{80,91,94,101} We chose the method reported by

Kane et al.,⁹⁹ which directly gives an ester substituent linked to a hydroxy group at the 1-position of the azulene scaffold. Thus, we decided to also study what kind of substituents could be introduced at the 1-position with the ester linker. Having a triflate group at the 1-position was not an option for us, since it causes selectivity problems with the halogen atom at the 6-position in the later stages of the synthesis. Thus, the azulene derivatives synthesized with this method are based on the 1-acyloxyazulene scaffold (Publication II).

The synthesis of the 6-chloro-substituted azulene scaffold was started from 4-chlorobenzaldehyde (**81**) (Scheme 5). 4-Chlorocinnamic acid (**82**) was synthesized in 92% yield using Knoevenagel condensation. In the hydrobromination of the double bond of **82**, we used more practical and commercially available HBr in glacial acetic acid instead of gaseous HBr of the reported method.⁹⁹ The reaction gave 98% yield, but the 4-day reaction time was needed for a high conversion. Kane et al. used previously diazomethane in the synthesis of diazo ketone **84** and we aimed at replacing it with the less explosive (trimethylsilyl)diazomethane. After converting **83** to the corresponding acyl chloride, (trimethylsilyl)diazomethane was added to generate the diazo ketone **84**, applying a method reported in the literature.¹³⁴ Optimization of the reaction conditions did not result only in the use of safer reagents but also in an improved overall yield of the diazo ketone **84** from cinnamic acid **82**. Our method gave a 77% yield in comparison to the 61% yield in the literature.⁹⁹



Scheme 5 *Synthesis of 1-acyloxyazulene scaffold. Reagents and conditions: (a) Malonic acid, piperidine, pyridine, 105 °C, 21 h, 92%; (b) 33% HBr in glacial acetic acid, rt, 96 h, 98%; (c) i) Oxalyl chloride, benzene, 65 °C, 18 h, ii) TMSCHN₂, MeCN, 0–4 °C, 24 h, 79%; (d) Rh₂(t-BuCO₂)₄, rt, 1.5 h, ii) Appropriate electrophile, DMAP, rt, 5 min, 62% (**85**), 40% (**86**), 33% (**87**), 40% (**88**), 14% (**89**), 51% (**90**), 25% (**91**), 32% (**92**).*

In the construction step of the azulene ring we investigated the possibility to vary the electrophile in substituting the 1-position using different activated carboxylic acid derivatives. First, a test reaction was run with acetic anhydride as electrophile to give 1-acetoxy-6-chloroazulene (**85**) in 62% yield (Scheme 5). Compound **85** has already been reported in the literature and the yield was consistent with the previously reported one.

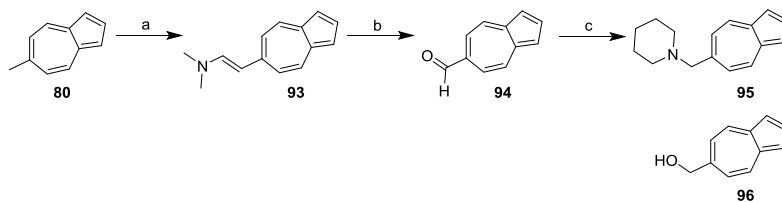
The reaction was also applied with succinic anhydride, which gave the carboxylic acid derivative **89**. The first attempt led to formation of an insoluble material, from which no product was isolated. Learning from this, the amount of succinic anhydride was reduced from five to one equivalent to produce **89** in 14% yield. The result suggests that the excess of the anhydride reacts further with the carboxyl group of **89** to form an insoluble residue. An additional explanation for the low yield could be the instability of the compound, which was found out after purification. The instability might be explained by the free carboxyl group, since the corresponding methyl ester derivative **90** is stable.

In addition to acid anhydrides, acyl chlorides and mixed anhydrides were used as electrophiles. Benzoyl chloride, nicotinoyl chloride and 2-thiophenecarbonyl chloride gave compounds **86**, **87** and **88** in 40%, 33% and 40% yield, respectively (Scheme 5). Mixed anhydrides were prepared from pivaloyl chloride and carboxylic acid derivatives and the method produced compounds **90** and **91** in 51% and 25% yield, respectively. In the case of **91**, the amount of mixed anhydride was reduced from five to two equivalents, after the first attempt had resulted in the formation of many side products that were difficult to separate from the desired product. The reaction was also tested with benzyl chloroformate as the electrophile resulting in the carbonate **92** in 32% yield. Using variable activated carboxylic acid derivatives we have shown that the method enables the functionalization of the 1-position with several ester- and carbonate-linked substituents. Of note, introduction of the desired functionality should be carried out in the azulene ring formation step, as instability of the 1-hydroxyazulene intermediate prevents later conversion of the ester group to other esters.

3.1.2 Functionalization of the 6-position

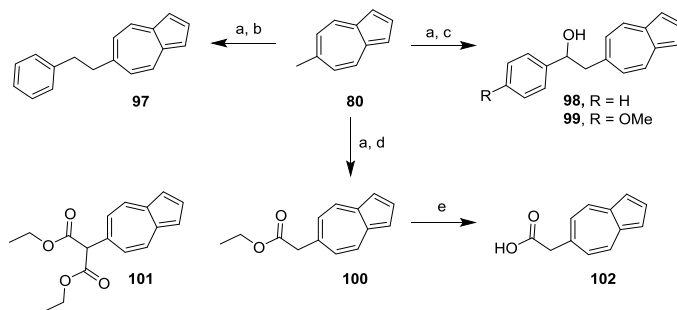
The applicability of the methyl group of 6-methylazulene was demonstrated with two different approaches, either via a formyl group or by deprotonation with a strong base (Publication I). We also showed that a chlorine atom in the 6-position can be used in palladium-mediated cross-coupling reactions (Publication II).

In the first approach, the methyl group was converted to a formyl group via an enamine by applying the method previously described for 1-methoxycarbonyl-4-formylazulene.¹³⁵ Starting from **80**, 6-formylazulene (**94**) was obtained in 68% yield over two steps (Scheme 6). Previously **94** has been synthesized from an acetal-protected 4-formylpyridine in an overall yield of 18%.¹³⁶ Our method gave **94** in an overall yield of 43% with the same number of synthetic steps and without the additional need of protecting groups. The versatility of the formyl group in further reactions was demonstrated by synthesizing the amino derivative **95** with reductive amination. The reaction gave **95** in 82% yield and also the corresponding alcohol **96** was isolated in 11% yield.



Scheme 6 Functionalization of the methyl group of 6-methylazulene via a formyl group. Reagents and conditions: (a) *N,N*-Dimethylformamide dimethyl acetal, DMF, 140 °C, 7 h; (b) NaIO₄, THF, H₂O, rt, 1 h, 68% from **80**; (c) Piperidine, NaBH(OAc)₃, DCM, rt, 5 h, 83% (**95**), 11% (**96**).

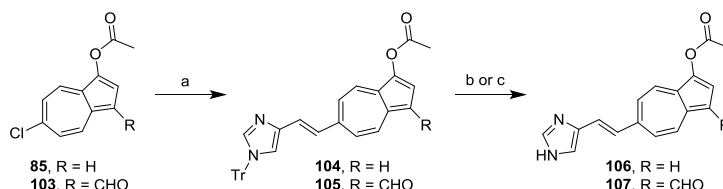
The second approach for 6-methyl group functionalization was the deprotonation of the methyl group of 6-methylazulene with a strong base,⁹⁰ which enables further reactions with various electrophiles. The use of *n*-butyllithium (*n*-BuLi) led to decomposition of the azulene scaffold, but the deprotonation with lithium diisopropylamide (LDA) produced the desired anion. Electrophiles were added directly to the reaction mixture after the azulene anion was formed. The reaction with benzaldehyde, 4-methoxybenzaldehyde, benzyl bromide and ethyl chloroformate gave the alcohols **98** and **99**, the phenyl derivative **97** and the ester **100** in 57%, 23%, 84% and 47% yields, respectively (Scheme 7). In each case a small amount ($\leq 16\%$) of 6-methylazulene was left unreacted. The differences in the observed yields are not likely caused by the variation of the reactivity between the used electrophiles but rather due to the reactivity of the formed products. The ester **100** is prone to deprotonation allowing the formed anion to react for second time with ethyl chloroformate to give diester **101**, which was obtained in 13% yield. Compounds **98** and **99** contain a hydroxy group in the benzylic position, and dehydration leads to a fully conjugated system. Dehydration in the basic reaction conditions is probable, although the dehydrated products could not be isolated from the reaction mixtures.



Scheme 7 Deprotonation of 6-methylazulene followed by reactions with various electrophiles. Reagents and conditions: (a) LDA, THF, 0 °C, 10 min and then rt, 20 min; (b) BnBr, -78 °C \rightarrow rt, 70 min, 84%; (c) Appropriate benzaldehyde, 0 °C, 10 min and then rt, 1.5 h, 57% (**98**), 23% (**99**); (d) EtO₂CCl, -78 °C \rightarrow 0 °C, 55 min, 47% (**100**), 13% (**101**); (e) NaOH, THF, H₂O, rt, 2 h, 96%.

Hydrolysis of the ester gave **102** in 96% yield (Scheme 7). Compound **102** has also been synthesized previously directly from the deprotonated 6-methylazulene and carbon dioxide in a good yield.⁹⁰ Our method is advantageous compared to the reported method in case the following synthetic step requires protection of the carboxyl group, since our method produces the ester directly.

The feasibility of the chlorine atom of the 1-acyloxyazulene scaffold in palladium-mediated cross-coupling reactions was demonstrated with Heck reaction (Publication II). Since imidazoles are important heterocycles in medicinal chemistry,¹³⁷ this moiety was introduced to the azulene scaffold. The Heck reaction was carried out for both the disubstituted azulene **85** and the trisubstituted azulene **103** with 1-trityl-4-vinyl-1*H*-imidazole, which gave compounds **104** and **105** in 59% and 67% yield, respectively (Scheme 8). Subsequently, the trityl groups were removed by trifluoroacetic acid (TFA). In the reaction mixture ethane-1,2-dithiol (EDT) was used as an additive to trap the formed trityl cation, which otherwise could have reacted with other nucleophiles, such as the 3-position of **106**. However, the use of EDT in the case of **105** led to the mixture of products, as **105** contains a formyl group, which was reactive towards EDT. Finally, the reaction was performed without EDT to give **107** in 92% yield.



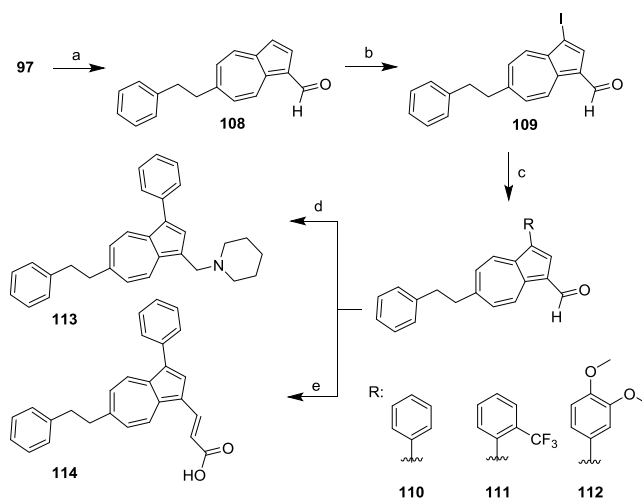
Scheme 8 Demonstration of the versatility of the chlorine atom in palladium-mediated cross-coupling reactions. Reagents and conditions: (a) 1-Trityl-4-vinyl-1*H*-imidazole, *P*(*t*-Bu)₃HBF₄, Et₃N, benzene, mw, 160 °C, 30 min, 59% (**104**), 67% (**105**); (b) TFA, ethane-1,2-dithiol, DCM, rt, 15 min, 91% (**106**); (c) TFA, DCM, rt, 30 min, 92% (**107**).

3.1.3 Functionalization of the five-membered ring

We investigated the possibilities to functionalize the 1- and 3-positions of the five-membered ring of azulene via synthetic handles. Formyl and iodine substituents are versatile synthetic handles, which easily can be introduced to these positions with Vilsmeier–Haack formylation^{88,138} and iodination with *N*-iodosuccinimide (NIS).¹³⁹ Due to this, we decided to study their use in further functionalization of the five-membered ring of azulene together with a chloromethylketone group, which is a feasible synthetic handle in the synthesis of heterocycles.¹⁴⁰ 6-Phenylethylazulene (**97**) and 1-acetoxy-6-chloroazulene (**85**) were selected as test compounds for scoping out these reactions (Publication I and II).

The monoformylated product **108** was synthesized from **97** in 88% yield after a fast Vilsmeier–Haack reaction (Scheme 9). Iodination of **108** with NIS gave the 1,3,6-

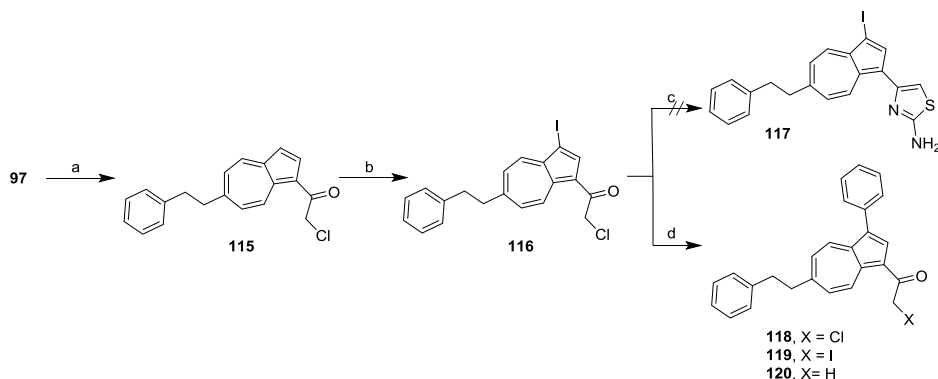
trisubstituted azulene **109** in high 97% yield. Noteworthy, the reactions should be done in this order, since the mono-iodinated product without a substituent at the 3-position is highly unstable and all our attempts to isolate it failed. Additionally, the iodination of the unsubstituted five-membered ring of azulene resulted in a mixture of mono- and di-iodinated compounds as well as unreacted starting material.



Scheme 9 Functionalization of the five-membered ring utilizing iodine and a formyl group as synthetic handles. Reagents and conditions: (a) (Chloromethylene)dimethyliminium chloride, DCM, 0 °C, 30 min, rt, 45 min, 88%; (b) NIS, DCM, 0 °C, 1 h, 97%; (c) An appropriate phenylboronic acid, Pd(dppf)Cl₂, BINAP, Cs₂CO₃, PhMe, mw, 110 °C, 1-2 h, 80% (**110**), 78% (**111**), 24% (**112**); (d) Piperidine, NaBH(OAc)₃, DCM, rt, 7 h, 92%; (e) Malonic acid, piperidine, pyridine, mw, 130 °C, 30 min, 39%.

Compound **109** with two useful synthetic handles can be considered as an important intermediate. The exploitation of the iodine atom in Suzuki cross-coupling reactions was demonstrated with three phenylboronic acids with different electronic properties. Applying the previously reported method¹⁴¹ the reaction gave compounds **110**, **111** and **112** in 80%, 78% and 24% yields, respectively (Scheme 9). Furthermore, the formyl group of **110** was converted to a carboxyl and an amino functionality. Knoevenagel condensation gave **114** in 39% yield and reductive amination gave **113** in 92% yield.

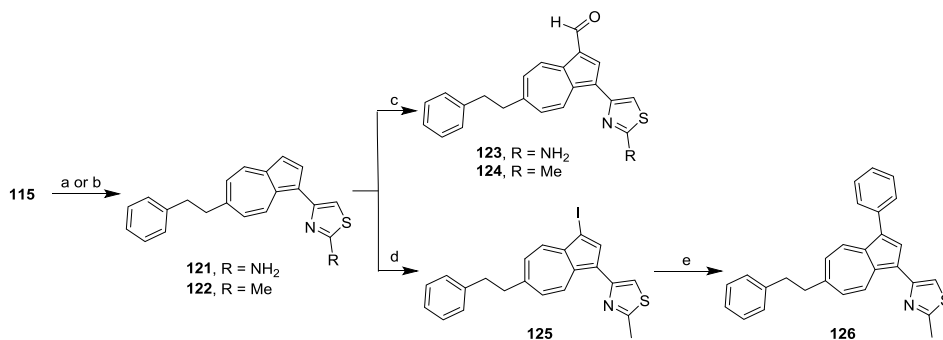
Next, we studied the possibilities to introduce a chloromethylketone group in the 1-position of the azulene scaffold. Our first attempt was to introduce it to 6-methylazulene with Friedel–Crafts acylation reaction using chloroacetyl chloride and AlCl₃. However, the reaction gave low yield, from 2% to 25%, most likely since both chlorine atoms in chloroacetyl chloride are reactive in Friedel–Crafts conditions. Better results were obtained applying a modified Vilsmeier–Haack reaction as described by Dragu et al.,¹⁴² by which the compound **115** was obtained in 73% yield (Scheme 10).



Scheme 10 Introduction of a chloromethylketone group in the 1-position and attempts to functionalize the five-membered ring via the intermediate **116**. Reagents and conditions: (a) 2-Chloro-*N,N*-diethylacetamide, POCl_3 , 1,4-dioxane, mw, 100°C , 2 h, 73%; (b) NIS, DCM, 0°C , 75 min, 97%; (c) Thiourea, EtOH, mw, 120°C , 5 min; (d) Phenylboronic acid, $\text{Pd}(\text{dppf})\text{Cl}_2$, BINAP, Cs_2CO_3 , PhMe, mw, 110°C , 1 h.

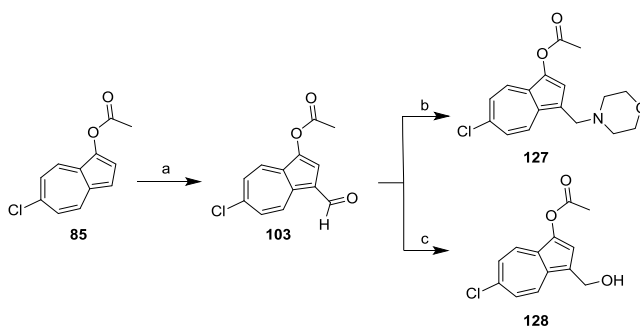
We studied the option that synthetic handles are introduced first to the 1- and 3-positions before reacting them further. Thus, an iodine atom was introduced to the 3-position of the chloromethylketone derivative **115** with NIS in 97% yield (Scheme 10). However, the further reactions of either the iodine or the chloromethylketone of compound **116** were problematic. Suzuki reaction with phenylboronic acid resulted in a mixture of compounds, of which two fractions were separated. The first fraction was a mixture of two compounds, which could not be separated from each other. However, NMR and HRMS analysis suggested that this fraction consisted of the desired product **118** and its analogue **119**, where the chlorine was replaced by an iodine (combined yield 25–30%). This mixture could nonetheless be used in e.g. formation of heterocycles, since both compounds are expected to give the same heterocycle. The second fraction contained the dehalogenated product **120** in 16% yield. Because of the low yield and the resulting mixture of compounds, we decided to try a different approach by first converting the chloromethylketone group of **116** to aminothiazole. Unfortunately, also this reaction gave several products and the desired one could not be isolated from the mixture.

Eventually, the best approach was to synthesize first the thiazole heterocycle from the chloromethylketone. Thiourea or thioacetamide were used as a reagent to obtain the aminothiazole **121** and the methylthiazole **122** in 92% and 86% yield, respectively (Scheme 11). Formylation of the 3-position was tested with both thiazole derivatives. The electron-rich aminothiazole ring caused selectivity problems in this reaction step and the desired product **123** could not be isolated from the mixture. Furthermore, **121** turned out to be relatively unstable. However, the methylthiazole derivative **124** was obtained in 56% yield. One reason for the moderate yield was the fact that approximately one-third of the starting material remained unreacted in the reaction. The iodination of the 3-position of **122** gave a 97% yield. Compound **125** was reacted in Suzuki coupling to produce **126** in 41% yield in the next step.



Scheme 11 Functionalization of the five-membered ring exploiting a chloromethylketone group. Reagents and conditions: (a) Thiourea, EtOH, mw, 120 °C, 30 min, 92% (**121**); (b) Thioacetamide, EtOH, mw, 120 °C, 1 h, 86% (**122**); (c) (Chloromethylene)-dimethyliminium chloride, DCM, 0 °C, 1 h, 56% (**124**); (d) NIS, DCM, 0 °C, 30 min, 97%; (e) Phenylboronic acid, Pd(dppf)Cl₂, BINAP, Cs₂CO₃, PhMe, mw, 110 °C, 1 h, 41%.

The 3-position of the 1-acyloxyazulene derivatives was substituted by introducing a formyl group as a synthetic handle (Publication II). 1-Acetoxy-6-chloroazulene (**85**) was used as a test substance as we studied the formylation reaction using both commercial and *in situ* prepared Vilsmeier–Haack reagent (Scheme 12). When the reagent was prepared from POCl₃ and DMF, the reaction temperature (room temperature or 90 °C) and the amount of POCl₃ (1.5–4 equiv) were varied. The yields of **103** were between 31% and 59% and the highest was obtained using two equivalents of POCl₃ at room temperature. Correspondingly the yield was 44% using the commercial reagent in DCM at 0 °C. We also noticed that there were variation in the yields when applying the same reaction conditions, which was likely caused by ester hydrolysis. To avoid this, NaHCO₃ was used instead of NaOH in the workup.



Scheme 12 Functionalization of the 3-position via a formyl group. Reagents and conditions: (a) POCl₃, DMF, rt, 45 min, 59%; (b) Morpholine, NaBH(OAc)₃, DCM, rt, 2.5 h, 53%; (c) BH₃·THF, THF, rt, 20 min, quant.

To demonstrate the feasibility of the formyl group, it was converted to an amino functionality by reductive amination, which gave **127** in 53% yield (Scheme 12). Furthermore, reduction of the formyl group with borane-THF complex gave **128** in quantitative yield, but it is unstable and decomposes at room temperature already within a few days.

3.2 Azulene-based compounds for targeting orexin receptors

In this work two series of compounds for targeting orexin receptors were designed and synthesized. With the first series, we aimed at finding azulene-based compounds, which act through the orexin receptors (Publication III). The second series of compounds was designed based on the biological results from the first one in order to find enhanced agonist activity on orexin receptors (Publication IV). The two series of compounds are discussed together in this chapter.

3.2.1 Designing the compounds for synthesis

We began the design of the azulene-based compounds using computational methods to give an idea on which azulene derivatives should be synthesized first. Without any clues from computational modeling, the chances of finding interesting bioactivities on orexin receptors only by attaching certain functionalities on the azulene scaffold are rather low, no matter how relevant the respective functionalities would be on the orexin-A peptide itself. We built a virtual library of di- and trisubstituted azulenenes (substituents in the 1-, 3- and 6-positions), which were accessible with our synthetic protocols for 1,3,6-trisubstituted azulenenes (Publications I and II). The selected azulene scaffold substituents had varying hydrogen-bonding properties, size, aromaticity, and linker lengths between the substituent and the scaffold. Eventually the virtual library consisted of 70 038 azulene-based compounds with molecular weight being below 500 g/mol.

The virtual library was then docked into the crystal structure of OX₂ receptor, based on the results of the previously developed pharmacophore model to discover orexin receptor ligands.⁵⁹ Similarities between the top-scoring compounds were examined by visual inspection. With this approach we noticed that the 1-position was often occupied by a benzoyl derivative and the 6-position by primary carboxamide, or the corresponding carboxylic acid. We then selected the compounds for synthesis in the first series from the visually examined set.

The results from the first series, which is described in detail in the section 3.2.3, guided us in the design of the second series of compounds. We were interested in identifying new azulene-based compounds, which would show better agonist activity on the orexin receptors than the best compounds of the first series. The most promising compounds **132** and **152** were used as a starting point for modifications. In addition to the enhanced activity, the aim was also to improve the pharmacokinetic properties of the

compounds, as the weak agonists from the first series are highly lipophilic and contain ester functionality that is sensitive to hydrolysis.

This time we designed the series so that either the benzoyl group in the 1-position was modified, or the ester group in the 6-position was replaced by an amide group. When designing the compounds, the ester and amide groups in the 6-position were linked directly to the azulene scaffold (like in **132**), since synthetic modifications of the ester linked with a methylene group (like in **152**) were expected to be problematic (see section 3.2.2). The planned modifications in the 1-position were attachment of polar substituents to the benzoyl group, or replacement of the benzoyl group with other aromatic groups, amide derivatives or oxoamide derivatives. We also aimed at synthesizing indole analogues to assess the significance of azulene scaffold for the observed biological activities.

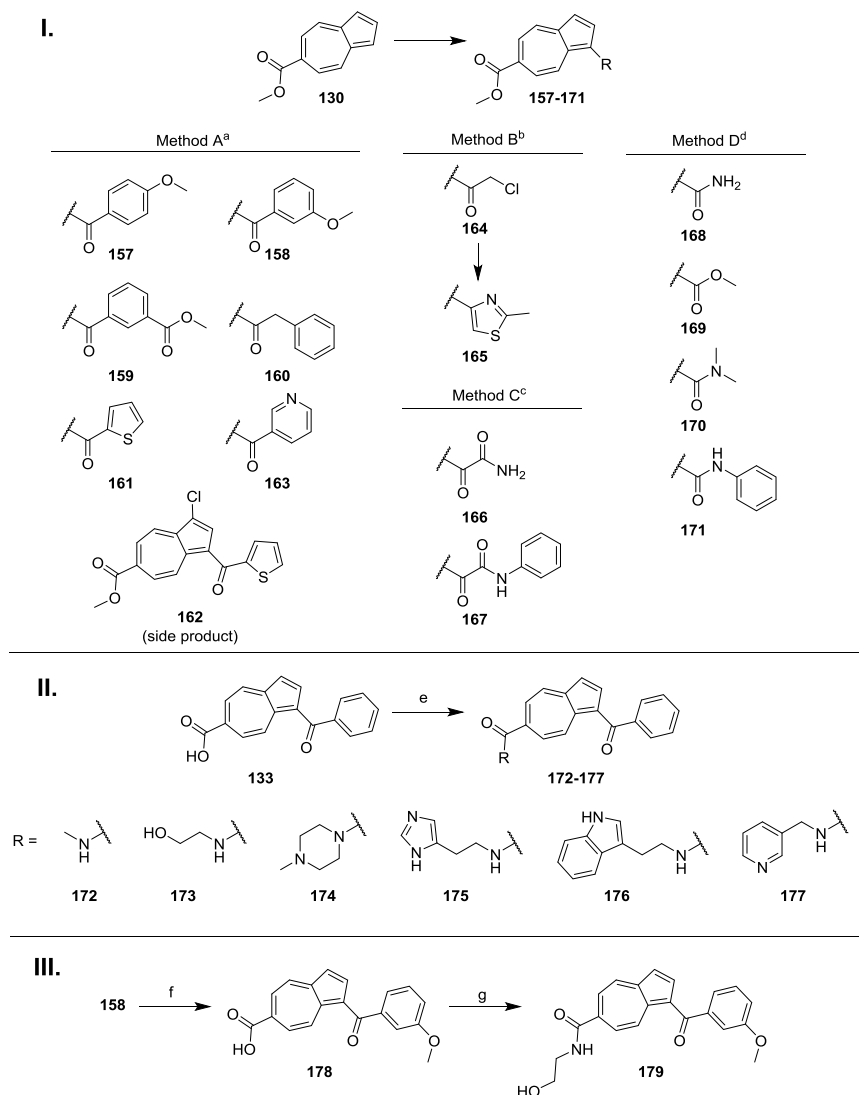
3.2.2 Synthesis of the designed compounds

The designed compounds were synthesized applying the protocols described in the section of 3.1. We also developed these synthetic methodologies further, as described in the following chapters, as well as synthesized certain indole analogues as reference compounds. The detailed synthetic descriptions can be found in the experimental sections of publications III and IV. The synthetic routes to access the first and second series of azulene-based compounds are shown in Scheme 13 and Scheme 14, respectively.

We investigated the oxidation of **94** to obtain the ester **130** with the method originally developed for benzaldehydes.¹⁴³ Our first attempt to synthesize the ester **130** directly from **94**, by stirring one equivalent of Oxone in methanol at room temperature for 17 h, ended up in formation of an insoluble solid, which appeared to be neither the desired product nor the starting material. To avoid this, we decided to oxidize the formyl group first to a carboxyl group, and methylate it afterwards. 6-Carboxyazulene (**129**) was synthesized by stirring the aldehyde **94** and Oxone in DMF at room temperature for 3 h in 48% yield. In this reaction, 32% of the starting material was also recovered. Extension of the reaction time did not increase the yield, even though the starting material was consumed almost completely. Finally, the methylation of **129** with iodomethane gave **130** in 96% yield.

In order to add the second benzoyl group to the five-membered ring of **131**, we first tried to apply the modified Vilsmeier–Haack conditions. However, in these reaction conditions the starting material decomposed and the desired product was not observed. Thus, Friedel–Crafts acylation was used instead, and the acylation of **131** with benzoyl chloride and AlCl₃ gave the trisubstituted azulene **136** in 87% yield. Applying the same conditions for compound **132** resulted in a low yield of a mixture of **137** and **138**. These two compounds were inseparable despite several purification attempts by flash chromatography. Similarly, the corresponding carboxylic acids **140** and **141** were not separable.

Of note, we also observed that the carboxyl group of **154** is prone to decarboxylation. Our attempts to convert it to the corresponding primary amide failed and only the decarboxylated product **155** was isolated in 85% yield.

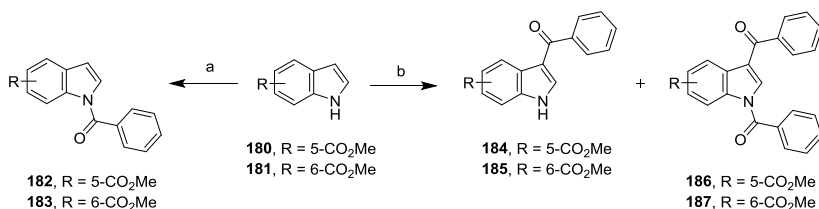


Scheme 14 *Synthesis of the designed azulene-based compounds (the 2nd series). Reagents and conditions: (a) An appropriate N,N-dimethylbenzamide, POCl₃, 1,4-dioxane, mw, 100 °C, 4–5 h, 22% (**157**), 18% (**158**), 44% (**159**), 31% (**160**), 29% (**161**), 0% (**163**); (b) i) 2-Chloro-N,N-diethylacetamide, POCl₃, 1,4-dioxane, mw, 100 °C, 2 h, 33%, ii) Thioacetamide, EtOH, mw, 120 °C, 30 min, 71%; (c) i) Oxalyl chloride, toluene, rt, 1 h, ii) An appropriate amine, DCM, 10–30 min, 64% (**166**), 50% (**167**); (d) i) Phosgene, toluene, 80 °C, overnight, ii) An appropriate amine, 0 °C, 15–30 min, 35% (**168**), 9% (**169**), 49% (**170**), 84% (**171**); (e) i) EDC·HCl, HOBT·H₂O, DMF, 0 °C, 15 min, rt, 1 h ii) An appropriate amine derivative, rt, 1–2.5 h, 79% (**172**), 85% (**173**), 88% (**174**), 49% (**175**), 81% (**176**), 80% (**177**); (f) NaOH, THF, MeOH, H₂O, rt, 30 min, 98%; (g) i) EDC·HCl, HOBT·H₂O, DMF, 0 °C, 15 min, rt, 1 h ii) Ethanolamine, rt, 1.5 h, 87%.*

Compounds **157–161** were synthesized with the modified Vilsmeier–Haack method and the yields were low or moderate (Scheme 14-I, Method A). The unreacted starting material was collected and it was used again in other reactions. However, the re-used starting material was not totally pure, because a small amount of 1-chloro-substituted starting material was formed in the reaction and it was not separable from the starting material with flash chromatography. Due to this, in the case of **161** also the side product **162** was isolated. We also tried to synthesize **163**, but the reaction failed and only insoluble solid was formed, most likely due to nucleophilic nitrogen atom in pyridine, which also reacts with the chloroiminium ion derivative. Furthermore, the modified Vilsmeier–Haack method was used in the synthesis of **164**, which was then converted to the thiazole **165** (Scheme 14-I, Method B).

Oxalyl chloride has been reported to react with the 1-position of azulene.¹⁴⁴ Compound **130** was first treated with oxalyl chloride at room temperature and then with an appropriate amine derivative to provide **166** and **167** in 64% and 50% yield, respectively (Scheme 14-I, Method C). We tried to utilize the same method in the synthesis of the amides in the 1-position by replacing oxalyl chloride with phosgene. However, phosgene was not as reactive as oxalyl chloride and the reaction did not proceed at the room temperature. Heating at 80 °C overnight, followed by the addition of an appropriate amine derivative gave the amides **168**, **170** and **171** in 35%, 49% and 84% yield, respectively (Scheme 14-I, Method D). Additionally, the ester **169** was obtained as a side product from the reaction of **168**, as methanolic ammonia was used in the reaction. The amides **172–177** and **179** were synthesized from the corresponding carboxylic acids **133** and **178** in good yields (Scheme 14-II and III).

Methyl indolecarboxylates were used as starting materials in the synthesis of indole analogues (Scheme 15). The 1-position of indole derivative was substituted with benzoyl group using benzoyl chloride in the presence of triethylamine. Compounds **182** and **183** were obtained in 59% and 22% yields, respectively. Compounds **184** and **185** were prepared following the same method, which was used for **131**. The method gave a mixture of compounds with the desired compounds being obtained only in 2–3% yield, even though the reaction time and the amount of *N,N*-dimethylbenzoylamine were varied. The major products were the trisubstituted indoles **186** and **187**, isolated in 23% and 48% yield, respectively.



Scheme 15 Synthesis of indole derivatives. Reagents and conditions: (a) Benzoyl chloride, Et₃N, DCM, rt, 23 h, 59% (**182**), 22% (**183**); (b) *N,N*-Dimethylbenzamide, POCl₃, mw, 100 °C, 1–3 h, 2% (**184**), 3% (**185**), 23% (**186**), 48% (**187**).

3.2.3 Evaluation of the biological activity

The first compound series in biological activity determination consisted of 33 azulene derivatives, of which four were tested as mixtures (Publication III). Sixteen of these compounds were the synthetic products inspired by virtual screening, and seven were their synthetic intermediates. Additionally, eight previously synthesized compounds were included in this series. The second compound series consisted of 21 azulene-based compounds and six indole-based compounds, respectively (Publication IV).

The methods to assess the binding of the compounds to orexin receptors and their agonist and potentiation effects are described briefly in the beginning of each section. More detailed information of the methods used can be found from the publications III and IV.

3.2.3.1 Orexin receptor binding

The binding of the compounds to the orexin receptors was evaluated at 10 μ M concentration with a competition binding assay in Chinese hamster ovary-K1 (CHO) cells heterologously expressing of human OX₁ and OX₂ receptors, using 1 nM [¹²⁵I]-orexin-A as the radioligand. Inhibitory constants (K_i) were determined using a Ca²⁺ elevation assay. The binding was evaluated only for the first compound series, as we focused on studying the agonist activity with the second series.

Twelve compounds (**86–88**, **91**, **92**, **107**, **127**, **133**, **145–147**, **155**) of the first series showed clear (inhibition >30%) and statistically significant inhibition of [¹²⁵I]-orexin-A binding to either one or both receptor subtypes (Table 2). K_i values were determined for the two most promising binders **127** and **147**, and they were in low micromolar range for both receptor subtypes.

Unexpectedly, six compounds (**132**, **134**, **142**, **144**, **152**, **153**) increased significantly the binding of [¹²⁵I]-orexin-A to OX₁ receptor in the binding assay. Similar effect has been noticed also previously with weak small-molecular agonists.⁵⁹ Additionally, the extracellular Ca²⁺ is known to stimulate the OX₁ receptor signaling and orexin-A binding to OX₁ receptor.^{145–147} The potentiation of orexin-A response to these compounds was studied further and will be discussed more in detail in section 3.2.3.3, but in short: **144** was the only compound to increase the [¹²⁵I]-orexin-A binding without inducing Ca²⁺ elevation or acting as a potentiator of orexin-A at 10 μ M concentration. Due to this, **144** was further examined by determining K_i values (Table 2) and the Ca²⁺ responses at concentrations exceeding 10 μ M (Figure 14).

Even though the number of the tested compounds is relatively low, the series of compounds is congeneric, which could allow preliminary structure-activity relationships (SARs) examination. However, our compound series contained both weak orexin receptor agonists and compounds potentiating the response of orexin-A to orexin receptors. Weak agonists and potentiators have an effect on the binding results, which hampers the interpretation of the binding results (see publication III for more information).

Table 2 *Screening results of the orexin receptor binding and activation of the first series compounds and K_i values for 127, 144 and 147.*

Compound	Inhibition (%) of [125 I]-orexin-A binding at 10 μ M ^a		K_i (μ M)		Ca ²⁺ elevation at 10 μ M (% of E_{\max} of orexin-A) ^b	
	OX ₁ R	OX ₂ R	OX ₁ R	OX ₂ R	OX ₁ R	OX ₂ R
147	40.7 \pm 13.0 *	47.2 \pm 6.8 *	4.8 \pm 1.8	3.6 \pm 1.1	1.6 \pm 0.7	0.7 \pm 0.7
127	24.5 \pm 10.7 *	46.7 \pm 10.7 *	7.6 \pm 2.0	8.6 \pm 2.8	0.4 \pm 0.3	-1.0 \pm 0.4
146	37.8 \pm 6.0 *	44.1 \pm 9.5 *	n.d.	n.d.	1.8 \pm 0.7	3.5 \pm 0.9
107	23.0 \pm 4.8 *	39.9 \pm 7.1 *	n.d.	n.d.	2.4 \pm 1.0	0.4 \pm 1.3
145	25.0 \pm 9.7 *	37.2 \pm 11.1 *	n.d.	n.d.	3.1 \pm 1.2	2.3 \pm 0.5
91	-4.5 \pm 2.5 *	36.0 \pm 7.6 *	n.d.	n.d.	1.6 \pm 0.2	-1.0 \pm 0.1
92	18.2 \pm 2.8 *	35.6 \pm 4.1 *	n.d.	n.d.	0.5 \pm 0.6	-2.7 \pm 0.7
86	0.1 \pm 12.9	34.3 \pm 9.1 *	n.d.	n.d.	0.1 \pm 0.7	-3.3 \pm 0.5
88	9.8 \pm 10.8	33.2 \pm 10.2 *	n.d.	n.d.	0.9 \pm 0.6	-2.7 \pm 0.8
133	11.0 \pm 16.5	32.9 \pm 12.0 *	n.d.	n.d.	2.2 \pm 1.5	2.3 \pm 2.6
87	-2.4 \pm 12.5	32.3 \pm 9.2 *	n.d.	n.d.	0.6 \pm 0.4	0.6 \pm 0.4
155	38.3 \pm 0.8 *	31.0 \pm 15.6	n.d.	n.d.	0.6 \pm 0.9	-0.4 \pm 0.6
131	7.2 \pm 17.2	28.9 \pm 8.5 *	n.d.	n.d.	4.2 \pm 2.1	4.9 \pm 1.5
139	-10.4 \pm 6.1	27.0 \pm 12.5	n.d.	n.d.	-1.4 \pm 1.1	-2.1 \pm 0.9
137/138 ^c	18.3 \pm 8.5 *	26.4 \pm 7.8 *	n.d.	n.d.	0.7 \pm 1.0	-0.1 \pm 0.1
140/141 ^c	19.9 \pm 11.3 *	25.6 \pm 8.8 *	n.d.	n.d.	-1.4 \pm 1.1	-3.2 \pm 0.4
90	8.0 \pm 7.0	24.3 \pm 4.2 *	n.d.	n.d.	0.7 \pm 0.4	-2.1 \pm 0.7
149	16.2 \pm 8.1 *	21.5 \pm 10.1 *	n.d.	n.d.	0.8 \pm 0.2	-0.2 \pm 0.6
143	8.4 \pm 12.3	19.1 \pm 9.6	n.d.	n.d.	1.5 \pm 0.2	-0.2 \pm 0.4
135	13.6 \pm 10.5	18.0 \pm 13.9	n.d.	n.d.	0.4 \pm 1.1	1.3 \pm 2.0
150	6.1 \pm 8.5	16.7 \pm 11.8	n.d.	n.d.	0.6 \pm 0.9	1.7 \pm 0.1
154	8.6 \pm 13.3	11.2 \pm 10.4	n.d.	n.d.	0.2 \pm 0.5	-1.1 \pm 0.6
148	8.5 \pm 8.9	1.8 \pm 3.4	n.d.	n.d.	0.8 \pm 0.1	-0.3 \pm 0.3
134	-81.5 \pm 18.6 *	1.0 \pm 5.8	n.d.	n.d.	1.1 \pm 0.7	3.0 \pm 0.8
153	-30.4 \pm 10.6 *	1.0 \pm 3.6	n.d.	n.d.	1.3 \pm 0.1	0.4 \pm 0.7
152	-37.7 \pm 8.1 *	-4.3 \pm 18.7	n.d.	n.d.	5.0 \pm 0.6	11.0 \pm 1.2
142	-34.9 \pm 2.1 *	-5.4 \pm 8.2	n.d.	n.d.	1.2 \pm 0.4	-0.3 \pm 0.1
136	-10.6 \pm 16.1	-14.5 \pm 12.4	n.d.	n.d.	-0.6 \pm 0.9	-1.5 \pm 0.4
144	-24.0 \pm 4.8 *	-16.9 \pm 16.4	7.5 \pm 0.9	4.9 \pm 2.0	-0.2 \pm 0.8	-1.0 \pm 1.4
156	-21.4 \pm 11.9	-20.4 \pm 11.2	n.d.	n.d.	0.3 \pm 0.6	0.1 \pm 0.2
132	-60.5 \pm 19.8 *	-47.4 \pm 11.4 *	n.d.	n.d.	6.1 \pm 0.6	12.4 \pm 0.5

*: $P < 0.05$; n.d. = not determined; ^a n = 3-4; ^b n = 2; ^c A 47:53 mixture of two compounds.

Based on the binding data, a slight trend seems to be that the compounds with the carboxyl group in the 6-position have better binding to the receptors, compared to the corresponding ester derivatives (e.g. compounds **145** vs. **146**, and **148** vs. **149**). Noteworthy, the opposite applies for the potentiators: the ester derivatives seem to be better potentiators than the corresponding carboxylic acid derivatives (e.g. **136** vs. **139**, and **153** vs. **154**). It might be that the carboxyl group in the 6-position enhances the binding of the compounds to the orthosteric orexin-A binding site or the potentiation effect of the ester derivatives hides the competition between the compound and [¹²⁵I]-orexin-A. Due to this, compounds with the ester group in the 6-position might bind better to OX₁ receptor than the binding data suggest.

The determination of *K_i* values for all compounds could help the interpretation of the binding results. However, we assessed them only for three compounds, as our main focus in this study was to find agonists not antagonists. Intensive research on orexin receptor antagonists have resulted in many reported antagonists, with the most potent ones having *K_i* values in the low nanomolar range (see section 1.1.2).

3.2.3.2 Agonist activity

The agonist activities of the compounds were screened in the Ca²⁺ elevation assay at 10 μM concentration, and the Ca²⁺ elevation was compared to the maximum response (*E_{max}*) of orexin-A. As part of the response, or even the whole response, could derive from the nonspecific response, the Ca²⁺ responses of the compounds were determined in the presence of an excess of a known orexin receptor antagonist, SB-334867¹⁴⁸ for OX₁R and TCS-1102¹⁴⁹ for OX₂R, respectively, to identify the magnitude of the nonspecific response. Subtracting the nonspecific response from the total Ca²⁺ elevation gave the Ca²⁺ elevation caused by the orexin receptor activation. For certain compounds, also the concentrated-dependent Ca²⁺ elevation was assessed using 10 μM, 18 μM and 32 μM concentrations, and 45 μM for **144** only. These compounds were also tested on control CHO-K1 cells to verify the orexin receptor specific response.

When screening the agonist activities of the first series, we noticed that three compounds, **131**, **132** and **152**, induced Ca²⁺ elevation, which was 5–12% of *E_{max}* of orexin-A, on both receptor subtypes (Table 2). The orexin receptor-mediated response was then determined from these three compounds. Compounds **132** and **152** induced approximately 5% Ca²⁺ elevation of *E_{max}* of orexin-A via OX₂ receptor, but **131** caused only nonspecific Ca²⁺ elevation at 10 μM concentration (Figure 13). These results indicate that **132** and **152** are weak OX₂ receptor agonists.

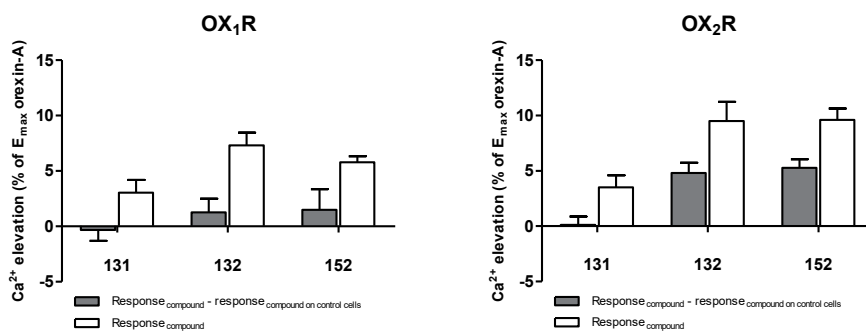


Figure 13 *Orexin receptor-mediated and total Ca²⁺ responses of 131, 132 and 152. (n = 3–4)*

The agonist activity screening results for the second series of azulene-based compounds and indole derivatives are shown in Table 3. The determined total Ca²⁺ responses were promising, as **167** and **177** induced Ca²⁺ elevation, which was 11–18% of E_{max} of orexin-A at 10 μ M, on both receptor subtypes. The Ca²⁺ responses of these compounds were noticeably higher than the responses determined with the best compounds of the first series. Unfortunately, after screening the second series of compounds in the presence of orexin receptor antagonists, most of the observed Ca²⁺ responses appeared to be nonspecific and not orexin receptor-mediated. Of note, none of the indole analogues showed orexin receptor-mediated Ca²⁺ elevation, and **182** was the only one to induce Ca²⁺ elevation at all. This was expected, since their closest azulene analogues, **131** and **136**, did not show orexin receptor activation either.

Altogether the concentration-dependent Ca²⁺ responses were determined for four compounds. Compound **132** was among the most promising compounds in the first series and used as a starting point for the second series of azulene-based compounds. In the second series, **177** and **179** showed the highest orexin receptor-mediated Ca²⁺ elevation at 10 μ M concentration. The selection of **144** is justified in section 3.2.3.1. All tested compounds showed concentration-dependent orexin receptor-mediated Ca²⁺ elevation (Figure 14).

Table 3 *Screening results of the orexin receptor activation of the second compound series.*

Compound	Ca ²⁺ elevation at 10 μ M (% of E _{max} of orexin-A) ^a			
	Total Ca ²⁺ responses		Orexin receptor-mediated Ca ²⁺ responses	
	OX ₁ R	OX ₂ R	OX ₁ R	OX ₂ R
157	0.6 \pm 0.8	1.2 \pm 1.9	0.1 \pm 0.8	0.4 \pm 1.5
158	0.6 \pm 0.0	0.1 \pm 0.6	-0.1 \pm 0.2	-1.0 \pm 0.5
159	0.5 \pm 0.3	0.7 \pm 0.8	-0.2 \pm 0.4	-0.2 \pm 0.7
160	3.4 \pm 1.1	4.9 \pm 1.4	-2.6 \pm 1.0	-1.5 \pm 1.3
161	2.0 \pm 1.9	-0.3 \pm 0.5	1.4 \pm 1.5	-1.1 \pm 0.3
162	-0.4 \pm 0.9	0.1 \pm 1.1	-1.4 \pm 0.6	-0.9 \pm 1.2
165	0.9 \pm 1.4	3.0 \pm 0.9	-1.5 \pm 1.2	1.3 \pm 0.9
166	-0.4 \pm 1.1	1.0 \pm 0.9	-1.6 \pm 0.7	-1.0 \pm 0.3
167	11.4 \pm 3.0	13.6 \pm 2.1	-4.3 \pm 1.2	1.5 \pm 1.5
168	-0.2 \pm 1.2	0.9 \pm 1.1	-1.6 \pm 0.9	-0.3 \pm 1.3
169	1.1 \pm 1.1	2.6 \pm 1.1	-2.3 \pm 0.7	-0.1 \pm 0.3
170	0.3 \pm 1.2	0.9 \pm 1.2	-0.8 \pm 0.8	-0.1 \pm 1.1
171	1.8 \pm 1.1	2.5 \pm 1.4	-0.4 \pm 0.3	1.1 \pm 1.0
172	1.2 \pm 2.3	0.6 \pm 3.4	-3.3 \pm 1.4	-6.6 \pm 1.8
173	2.5 \pm 0.6	5.7 \pm 1.1	0.1 \pm 0.4	0.7 \pm 1.0
174	1.2 \pm 1.0	2.6 \pm 1.3	-0.7 \pm 0.3	-1.7 \pm 1.5
175	2.3 \pm 0.3	0.1 \pm 1.3	0.3 \pm 0.9	-1.6 \pm 0.2
176	2.7 \pm 0.4	2.6 \pm 0.6	0.4 \pm 0.4	-1.3 \pm 0.6
177	11.1 \pm 0.2	17.7 \pm 1.3	2.2 \pm 0.1	0.1 \pm 2.4
178	0.1 \pm 0.4	0.8 \pm 0.4	-0.7 \pm 0.5	-0.7 \pm 0.9
179	5.8 \pm 0.3	8.4 \pm 1.3	1.0 \pm 0.3	3.1 \pm 1.9
182	0.9 \pm 0.3	4.6 \pm 0.6	-7.0 \pm 3.0	-1.1 \pm 2.5
183	-1.2 \pm 0.2	0.2 \pm 0.8	-3.7 \pm 1.5	-2.1 \pm 0.4
184	-0.6 \pm 0.3	0.5 \pm 0.3	-2.1 \pm 0.6	-1.0 \pm 0.5
185	-0.5 \pm 0.4	0.8 \pm 0.7	-2.5 \pm 0.4	-0.2 \pm 0.3
186	-1.1 \pm 0.5	0.8 \pm 0.8	-4.0 \pm 1.3	-2.1 \pm 1.5
187	-0.7 \pm 0.2	1.2 \pm 0.8	-4.1 \pm 1.4	-1.7 \pm 1.42

^a n = 3–4.

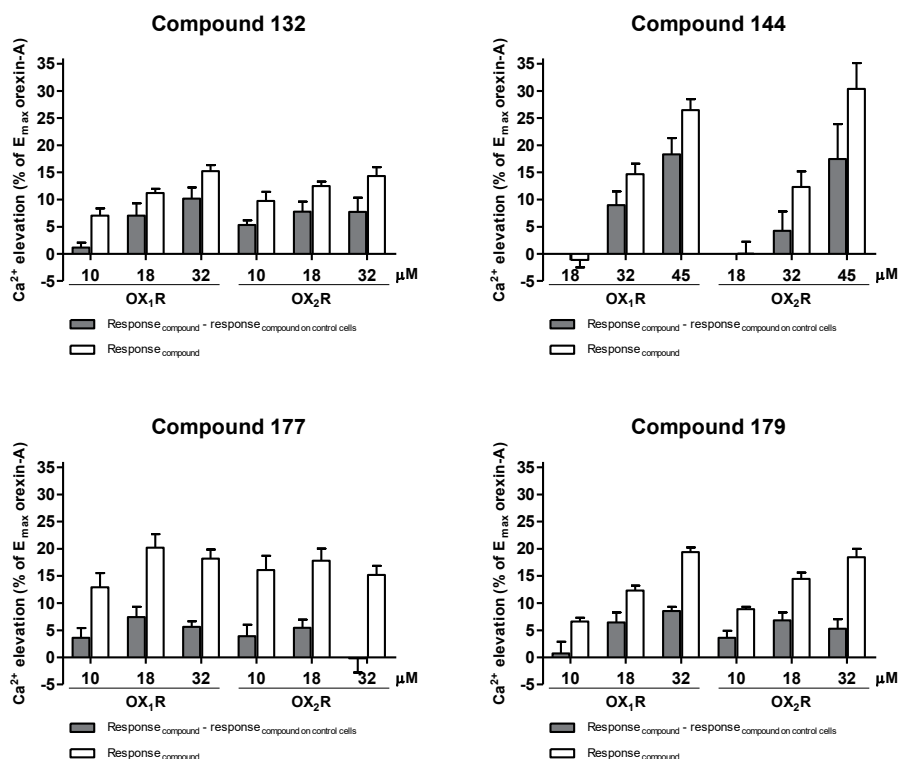


Figure 14 Orexin receptor-mediated and total Ca^{2+} responses of **132**, **144**, **177** and **179** at three different concentrations. ($n = 3-6$)

In this study, we have identified novel azulene-based weak orexin receptor agonists. Compounds **132** and **152** from the first compound series gave the best OX_2 -mediated Ca^{2+} responses at 10 μM . Compound **179** was the most promising one in the second series, and its specific Ca^{2+} responses at tested concentrations were of similar magnitude (slightly lower) than those of **132**. Furthermore, **179** is not as hydrophobic and it possesses improved solubility compared to **132** or **152**. Additionally, **144** showed orexin receptor-mediated Ca^{2+} elevation only at the highest concentrations (32 μM and 45 μM), but unlike **132**, **177** and **179**, the trend of the responses was clearly increasing at the highest concentrations. Altogether, **144** showed the highest determined Ca^{2+} responses in this study. At 45 μM concentration it induced Ca^{2+} responses, which were approximately 18% of the E_{max} for orexin-A, on both receptor subtypes.

Since only a few synthesized compounds showed agonist activity, and for the ones that did, the activity was weak. Therefore, it is difficult to make conclusions regarding the structure-activity relationships from the available data. Even though the identified agonist activities are weak, the compounds increase the knowledge of molecules acting as orexin

receptor agonists. Currently only one series of reported orexin receptor agonists are clearly more potent than our new compounds.

3.2.3.3 Potentiation of orexin-A response

The potentiation effect was studied by assessing the Ca^{2+} responses of the compounds in the presence of 0.02–0.03 nM orexin-A (corresponding to EC_{20} of the orexin-A) at 10 μM concentration. We used controls (10 nM or 30 nM ATP) to mimic the Ca^{2+} effect of the tested compounds, as changes in intracellular Ca^{2+} levels might potentiate the orexin-A response. To prove that the potentiation effect is transmitted through the orexin receptors, the effects of compounds on the actions of ATP in orexin receptor-expressing cells were tested with assay set-up identical to the one used with orexin-A. ATP causes a Ca^{2+} response similar to orexin-A, but the response is not orexin receptor-mediated.

The potentiation effect was studied with twelve compounds from the first series: six of them (**132**, **134**, **142**, **144**, **152**, **153**) increased significantly the binding of [^{125}I]-orexin-A to OX_1R in the binding assay, and other six compounds (**131**, **133**, **136**, **139**, **154**, **156**) potentiated [^{125}I]-orexin-A binding to OX_1R on some of the individual experiments. With the exception of **144**, all tested compounds potentiated the observed orexin-A response at least 1.4-fold on OX_1 receptors at 10 μM concentration (Figure 15). When comparing the potentiation to the controls, the effect of eight compounds (**133**, **134**, **136**, **139**, **142**, **153**, **154**, **156**) was significant, and **153** was the only one showing potentiation also on OX_2 receptors (ca. 1.3-fold). The potentiation induced by the compounds **131**, **132** and **152** was not significant when compared to the control (30 nM ATP), indicating these compounds induced an indirect potentiation of orexin-A response by increasing the intracellular Ca^{2+} levels. However, the detection of the potentiation effect might be too challenging in the case the compound induces Ca^{2+} elevation itself as well.

We evaluated further the three most promising OX_1 receptor potentiators **136**, **142**, **153** by assessing their concentration-dependency effects (Figure 15). Compound **153** appeared to be best potentiator with increasing the orexin-A response 1.4-fold at 5 μM and 2.0-fold at 10 μM concentrations. The detected potentiation effects are most likely orexin receptor-mediated, as the compounds did not affect the ATP response in OX_1 receptor-expressing cells (Figure 15).

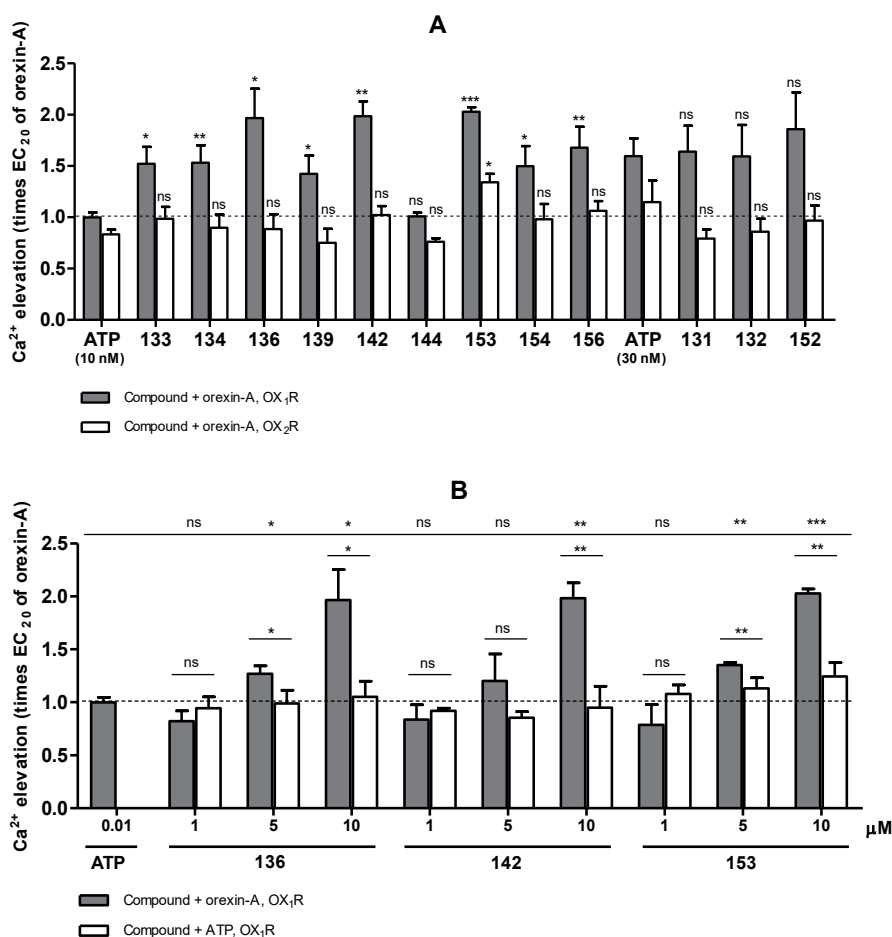


Figure 15 *A. The effects of the compounds from the first series at 10 μM concentration on Ca²⁺ responses elicited by 0.02–0.03 nM orexin-A. Controls, 10 nM or 30 nM ATP, mimicked the Ca²⁺ effects of the tested compounds. 10 nM ATP was used for 133, 134, 136, 139, 142, 144, 153, 154, 156, and 30 nM ATP for 131, 132, 152. B. The effects of 136, 142, 153 at three different concentrations on Ca²⁺ responses elicited by 0.02–0.03 nM orexin-A or 60 nM ATP (corresponding to the EC₂₀ of each ligand). ns = not significant ($P > 0.05$), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; $n = 3–4$.*

In the case of the second compound series, we tested the potentiation effect of all six indole derivatives and three azulene-based compounds (**157**, **158**, **178**). The azulene derivatives were selected as close but more hydrophilic analogues compared to the first series compounds, **131** and **133**, which also showed some potentiation of the orexin-A response to OX₁R, even though the effect of **131** might be indirect.

Four of six indole derivatives (**182**, **185**, **186**, **187**) potentiated the Ca^{2+} response to orexin-A on OX_1R (Figure 16). Compound **182**, an indole analogue of **131**, gave the best response, but when the results were compared to controls, the effect of **182** was not significant. Compound **182** induces nonspecific Ca^{2+} elevation similarly to **131**, and the effect is most likely indirect. Compounds **186** and **187** gave the best significant responses (1.7-fold), not surprisingly, as their azulene analogue **136** was one of the most promising potentiator of the first series. Furthermore, all three tested azulene derivatives of the second series showed potentiation of orexin-A responses on OX_1R , but the effect was not as good as the best compounds in the first series.

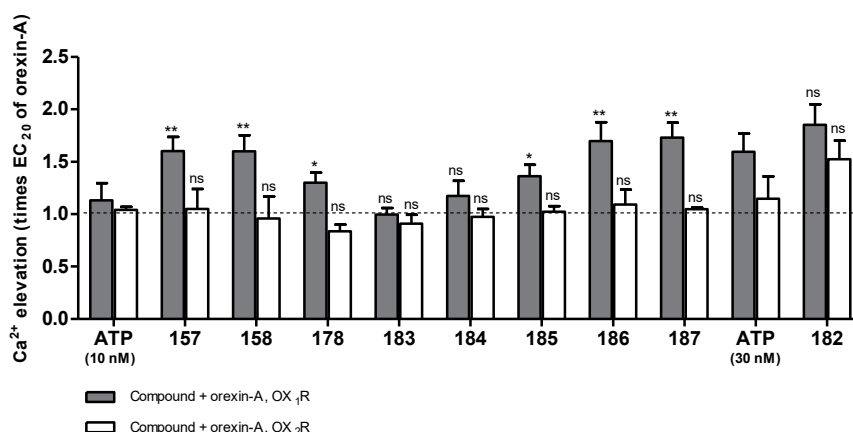


Figure 16 The effects of indole derivatives and three azulene-based compounds from the second series at 10 μM concentration on Ca^{2+} responses elicited by 0.02–0.03 nM orexin-A. 10 nM ATP was used as a control for all compounds, except for **182**. ns = not significant ($P > 0.05$), * $P < 0.05$, ** $P < 0.01$; $n = 3$ –4.

As pointed out in the section 3.2.3.1, the methyl ester group in the 6-position of azulene seems to be preferred over the carboxyl group for the potentiation effect of orexin-A. Furthermore our results demonstrate that when azulene scaffold is replaced by indole, these analogues have similar effects on the Ca^{2+} responses to orexin-A on OX_1 receptors (e.g. **136** vs. **186**, and **131** vs. **182**).

The orexin-A potentiation effect of the compounds can be considered as the most interesting finding in the biological evaluation of the synthesized azulene derivatives, since only one series of orexin receptor potentiators has been reported before us.⁶⁰ The observed potentiation effect of our best compounds was similar to the previously reported potentiators. However, the results are not fully comparable, as the evaluation methods differ from each other.

4 Summary and conclusions

This thesis describes the design, synthesis and biological evaluation of novel azulene-based compounds for targeting orexin receptors. Our approach was to develop general regioselective synthetic protocols for trisubstituted azulenes possessing the desired 1,3,6-trisubstitution pattern. We built a virtual library from chemically diverse azulene-based compounds that can be synthesized with the developed methods. By doing so we avoided getting a virtual screening hitlist, which contains synthetically useless compounds, or compounds whose synthesis would require several routes.

To access the differently substituted azulenes, the study was started by developing two general synthetic routes for 1,3,6-trisubstituted azulenes. Both methods began from inexpensive and readily available starting materials, and the azulene scaffold was formed first. We optimized the previously reported synthetic methods to obtain the azulene scaffold in higher yields. In our methods, the regioselective substitution of the azulene scaffold with versatile synthetic handles allows easy variation of the substituents. The efficiency of the synthetic routes was demonstrated with test substances, whose synthesis gave good overall yields.

The virtual library was docked into the crystal structure of OX₂R. The first series of compounds for biological activity determination was selected and synthesized after visual inspection of the virtual screening results. Most of the selected compounds were synthesized via the developed methods. The applicability of the synthetic methods with other top-scoring compounds was not studied, since in the second compound series we focused on further development of the first series hits. However, already the synthesis of the two compound series highlights the efficiency of the synthetic methods.

Altogether, the biological activity towards orexin receptors was determined from 54 azulene-based compounds and six indole derivatives. The main focus of this study was to discover orexin receptor agonists based on the azulene scaffold. We identified five novel azulene derivatives (**132**, **144**, **152**, **177**, **179**) as weak orexin receptor agonists (Figure 17). The first series hits, **132** and **152**, were highly lipophilic and we aimed at reducing the lipophilicity in the second compound series. As a result, we obtained **179** with improved solubility and retained agonist activity.

We identified **144** with the best Ca²⁺ response among all the tested compounds, despite it showed agonist activity only at the two highest concentrations. The detected Ca²⁺ elevation was approximately 18% of the E_{max} of orexin-A on both receptor subtypes. Thus, it is possible that also other compounds in our series behave similarly, showing agonist activity at higher concentrations than those used in the screening. Although we did not discover highly potent agonists in this study, our results extend the knowledge of small-molecular activating ligands for orexin receptors. During the agonist development, we also identified novel antagonists of the orexin receptors. The two most promising compounds, **127** and **147**, had K_i values in the low micromolar range (Figure 17).

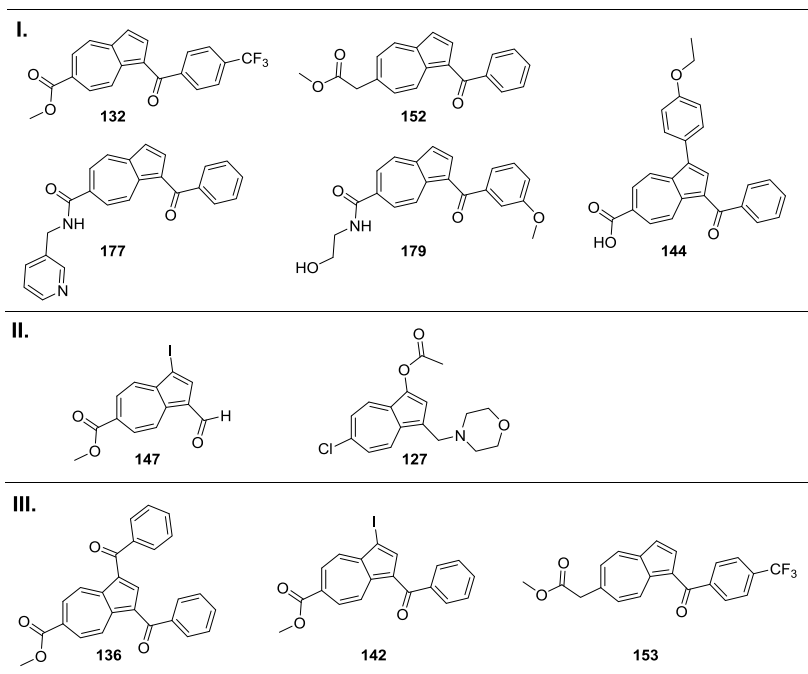


Figure 17 The most promising (I) agonists, (II) antagonists and (III) potentiators identified in this study.

The most interesting result in the biological evaluation was a set of azulene derivatives potentiating the response of orexin-A on OX₁ receptor. Three compounds (**136**, **142**, **153**) showed concentration-dependent potentiation and they increased the orexin-A response at 10 μ M two-fold, which is comparable with the potentiation effect observed with the only previously reported orexin receptor potentiators (Figure 17). Small-molecular potentiators of orexin receptor signaling could have similar applications than agonists in conditions caused by low levels of endogenous orexin peptides. Further research is needed to better understand the potentiation phenomenon, and to develop more effective potentiators.

The biological responses of the indole derivatives resembled those of the corresponding azulene analogues. Indoles **186** and **187** (analogues of azulene derivative **136**) potentiated the effects of orexin-A, but none of the indole derivatives induced orexin receptor-mediated Ca²⁺ elevation. The potentiation effect of the azulene **136** seemed slightly better compared to its indole analogues, with two possible explanations. Either the spatial arrangement of the substituents on the azulene scaffold is more favorable than on the indole scaffold, or the azulene ring itself has stronger interactions with the target than indole. Altogether these results support the idea that azulene can be considered as the isostere of indole.

Identification of novel agonists, antagonists and potentiators on orexin receptors justifies the strategy followed in the study. To further evaluate the success of the virtual screening and the applicability of the developed protocols in the synthesis of the virtual

library compounds, more top-scoring compounds should be synthesized. The biological activities of the discovered azulene-based compounds are still relatively weak, which emphasizes the need of more potent compounds with improved pharmacokinetic properties. In addition, new compounds would be essential for establishment of the structure-activity relationships.

To best of our knowledge, azulene-based compounds have not been studied on the orexin receptors previously. Thus, our azulene-based compounds open an unexplored chemical space and provide a new, interesting starting point for the development of small-molecular compounds acting through the orexin receptors. Our results also encourage medicinal chemists to use the azulene scaffold unprejudicedly in drug discovery.

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